# PCT

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : C07K 7/10, A61K 37/02 | A1 | (11) International Publication Number: WO 93/13128 |
|---|---|---|
| | | (43) International Publication Date: 8 July 1993 (08.07.93) |

(21) International Application Number: PCT/US92/11349

(22) International Filing Date: 30 December 1992 (30.12.92)

(30) Priority data:
07/814,759    30 December 1991 (30.12.91)   US

(60) Parent Application or Grant
  (63) Related by Continuation
  US                    07/814,759 (CIP)
  Filed on       30 December 1991 (30.12.91)

(71) Applicant *(for all designated States except US)*: NEUREX CORPORATION [US/US]; 3760 Haven Avenue, Menlo Park, CA 94025-1012 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : JUSTICE, Alan [US/US]; 1280A Poplar Avenue, Sunnyvale, CA 94086 (US). SINGH, Tejinder [US/US]; 1537 Louisa Court, Palo Alto, CA 94303 (US). GOHIL, Kishor, Chandra [GB/US]; 6217 Plymouth Avenue, Richmond, CA 94805 (US). VALENTINO, Karen, L. [US/US]; Two Elm Street, No. 202, San Carlos, CA 94070 (US). MILJANICH, George, P. [US/US]; 3623 Glenwood Avenue, Redwood City, CA 94062 (US).

(74) Agent: STRATFORD, Carol, A.; Law Offices of Peter J. Dehlinger, P.O. Box 60850, Palo Alto, CA 94306 (US).

(81) Designated States: AU, CA, HU, JP, KR, NO, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
  *With international search report.*
  *Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: METHODS OF PRODUCING ANALGESIA AND ENHANCING OPIATE ANALGESIA

(57) Abstract

Methods of producing analgesia and enhancing opiate analgesia are disclosed. The methods include administering a TVIA (SNX-185) or MVIIA (SNX-111) omega-conopeptide, or derivative thereof which is characterized by its ability to (a) inhibit voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) bind to omega conopeptide MVIIA binding sites present in neuronal tissue. Also disclosed is a novel omega conotoxin peptide derivative effective in producing analgesia.

*1*

## METHODS OF PRODUCING ANALGESIA
## AND ENHANCING OPIATE ANALGESIA

### 1.  Field of the Invention

5       The present invention relates to methods of producing analgesia and of enhancing opiate analgesia, particularly in the treatment of pain and neuropathic pain.

10   **2. References**

.Basilico, L., Parenti, M., Frevola, L., and Giagnon, G. (1989). Pharmacol. Res. 21:65-66.

Bennett, G. J. and Xie, Y.-K. (1988) Pain 33: 87-107.

15       Bennett, J. P. et al. (1983)  Neurotransmitter Receptor Binding pp. 61-89; Raven Press, New York.

Ben-Sreti, M. M., Gonzalez, J.P. and Sewell, R. D. E. (1983) Eur. J. Pharmacol. 90: 385-391.

Contreras, E., Tamayo, L., and Amigo, M. (1988)

20   Eur. J. Pharmacol. 148: 463-466.

Dixon, W.J. (1976) Ann. Rev. Pharmacol. Toxicol. 20: 441-462.

Fitzgerald, M. (1989) TINS 12(3): 86-87. ·

Gray, W., Olivera, B., and Cruz, L. (1988), Annual

25   Review of Biochemistry 57:665-700.

Hartley, D. and Choi, D. (1989), The Journal of Pharmacology and Experimental Therapeutics 250:752-758.

Kenakin, T. P. (1987) Pharmacologic Analysis of Drug-Receptor Interaction, Raven Press, NY.

30       Kim, S.H. and Chung, J.M.  (1992) Pain 50: 355-363.

McCleskey, E.W. et al., Proc. Natl. Acad. Sci. USA 84:4327-31 (1987).

McGeer, P.L., Eccles, J.C. and McGeer, E. G.

35   (1987) Molecular Neurobiology of the Mammalian Brain Plenum Press, NY.

2

Nowycky, M.C., Fox, A.P., and Tsien, R.W., Nature (London), 316:440-443 (1985).

Olivera, B., McIntosh, M., Cruz, L., Luque, F., and Gray, W. (1984), Biochemistry 23:5087-5090.

5    Paxinos, G., and Watson, C. (1986). The Rat Brain in Stereotaxic Coordinates, 2nd Edition.

Ritchie, J. M. and Greene, N. M. (1990) in Goodman and Gilman's The Pharmacological Basis of Therapeutics, (Eighth Edition) Pergamon Press (New York) Chapter 15,
10   pp. 311-331.

Sher, E. et al. (1991) FASEB J. 5: 2677-2683.

Takemura, M., et al. (1988). Neuroscience Res. 5:405-416.

Yaksh, T.L. and Noueihed, R. (1985) Ann. Rev.
15   Pharmacol. Toxicol 25: 433-462.

Yaksh, T.L., and Rudy, T.A. (1976) Physiol. Behav. 17: 1031-1036.

Yaksh, T.L., Yamamoto, T, and Myers, R.R. (1992) in Hyperalgesia and Allodynia (Willis, W.D., editor),
20   Raven Press, New York, Chapter 20, pp. 245-258.


3.   Background of the Invention

Chronic or intractable pain, such as may occur in conditions such as bone degenerative diseases and
25   cancer, is a debilitating condition which is treated with a variety of analgesic agents, and often opioid compounds, such as morphine.

In general, brain pathways governing the perception of pain are still incompletely understood,
30   sensory afferent synaptic connections to the spinal cord, termed "nociceptive pathways" have been documented in some detail. In the first leg of such pathways, C- and A-fibers which project from peripheral sites to the spinal cord carry nociceptive signals.
35   Polysynaptic junctions in the dorsal horn of the spinal

3

cord are involved in the relay and modulation of
sensations of pain to various regions of the brain,
including the periaqueductal grey region (McGeer).
Analgesia, or the reduction of pain perception, can be
5    effected directly by decreasing transmission along such
nociceptive pathways. Analgesic opiates are thought to
act by mimicking the effects of endorphin or enkephalin
peptide-containing neurons, which synapse
presynaptically at the C- or A- fiber terminal and
10   which, when they fire, inhibit release of
neurotransmitters, including substance P. Descending
pathways from the brain are also inhibitory on C- and
A- fiber firing.

     Certain types of pain have complex etiologies.
15   For example, neuropathic pain is generally a chronic
condition attributable to injury or partial transection
of a peripheral nerve. This type of pain is
characterized by hyperesthesia, or enhanced sensitivity
to external noxious stimuli. The hyperesthetic
20   component of neuropathic pain does not respond to the
same pharmaceutical interventions as does more
generalized and acute forms of pain.

     Opioid compounds (opiates) such as morphine, while
effective in producing analgesia for many types of
25   pain, are not always effective, and may induce
tolerance in patients. When a subject is tolerant to
opioid narcotics, increased doses are required to
achieve a satisfactory analgesic effect. At high
doses, these compounds produce side effects, such as
30   respiratory depression, which can be life threatening.
In addition, opioids frequently produce physical
dependence in patients. Dependence appears to be
related to the dose of opioid taken and the period of
time over which it is taken by the subject. For this
35   reason, alternate therapies for the management of

4

chronic pain are widely sought after. In addition, compounds which serve as either a replacement for or as an adjunct to opioid treatment in order to decrease the dosage of analgesic compound required, have utility in the treatment of pain, particularly pain of the chronic, intractable type.

Although calcium blocking agents, including a number of L-type calcium channel antagonists, have been tested as adjunct therapy to morphine analgesia, positive results are attributed to direct effects on calcium availability, since calcium itself is known to attenuate the analgesic effects of certain opioid compounds (Ben-Sreti). EGTA, a calcium chelating agent, is effective in increasing the analgesic effects of opioids. However, results from tests of calcium antagonists as adjunct therapy to opioids have been contradictory; some L-type calcium channel antagonists have been shown to increase the effects of opioids, while others of these compounds have been shown to decrease opioid effects (Contreras).

U.S. Patent No. 5,051,403 describes the use of omega-conopeptides having defined binding/inhibitory properties in the treatment of ischemia-related neuronal damage. In the present invention, it has been found that omega-conopeptides having related inhibitory and binding activities produce analgesia. In addition, these compounds also enhance the analgesic effects of sub-threshold levels of opioid compounds in mammalian subjects.

4. <u>Summary of the Invention</u>

The invention includes, in one aspect, a method of enhancing the analgesic effect produced by administration of opiates to a mammalian subject. In another aspect, the invention includes a method of producing analgesia in a mammalian subject. The

5

methods include administering to the subject, an omega conopeptide which is either TVIA (SNX-185), MVIIA (SNX-111) or a derivative thereof which is effective to (a) to inhibit voltage-gated calcium channels selectively

5    in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) to bind to omega conopeptide MVIIA binding sites present in neuronal tissue. The omega-conopeptide is administered at a

10   dose effective to produce analgesia or to enhance the analgesic effect of the opiate, as described herein.

In preferred embodiments, the activities of the omega-conotoxin in calcium-channel inhibition and in binding to the MVIIA binding site are within the ranges

15   of such activities of omega-conotoxins MVIIA and TVIA, and the omega-conopeptide is administered intrathecally. In another preferred embodiment, the omega conotoxin derivative is SEQ ID NO: 30.

In another aspect, the invention includes the

20   omega conotoxin peptide having the sequence SEQ ID NO: 30.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is

25   read in conjunction with the accompanying drawings.

## Brief Description of the Drawings

Figure 1 shows primary sequences of several natural omega-conopeptides, MVIIA/SNX-111 (SEQ ID NO:

30   01), MVIIB/SNX-159 (SEQ ID NO: 02), GVIA/SNX-124 (SEQ ID NO: 03), GVIIA/SNX-178 (SEQ ID NO: 04), RVIA/SNX-182 (SEQ ID NO: 05), SVIA/SNX-157 (SEQ ID NO: 06), TVIA/SNX-185 (SEQ ID NO: 07), SVIB/SNX-183 (SEQ ID NO: 08), and MVIIC/SNX-230 (SEQ ID NO: 29), and SNX-231

35   (SEQ ID NO: 21);

6

Figure 2 shows several analog omega-conopeptides SNX-190 (SEQ ID NO: 09), SNX-191 (SEQ ID NO: 10), SNX-193 (SEQ ID NO: 11), SNX-194 (SEQ ID NO: 12), SNX-195 (SEQ ID NO: 13), SNX-196 (SEQ ID NO: 14), SNX-197 (SEQ ID NO: 15), SNX-198 (SEQ ID NO: 16), SNX-200 (SEQ ID NO: 17), SNX-201 (SEQ ID NO: 18), SNX-202 (SEQ ID NO: 19), SNX-207 (SEQ ID NO: 20), SNX-236 (SEQ ID NO: 30) and their relationships to SNX-111 (SEQ ID NO: 01), SNX-185 (SEQ ID NO: 07) or SNX-183 (SEQ ID NO: 08);

Figures 3A-3D show voltage-gated calcium current traces induced by a voltage step from -100 or -80 mV to -20 mV in untreated N1E-115 neuroblastoma cells (3A) and in neuroblastoma cells exposed to increasing concentrations of OCT MVIIA (SNX-111) (3B-3D);

Figure 4 plots the percent inhibition of peak inward calcium currents in neuroblastoma cells as a function of OCT MVIIA (SNX-111) (solid triangles) and OCT GVIA (SNX-124)(solid circles);

Figure 5A shows voltage-gated calcium current traces induced by a voltage step from -70 to -20 mV in human neuroblastoma cells (IMR-32) in the absence (lower trace) and presence (upper tracing) of 150 nM SNX-111;

Figures 5B and 5C show plots of absolute values of peak inward current measured every 15 seconds in IMR-32 cells, elicited by pulses from -70 to 0 or -10 mV, versus time, where addition of compounds SNX-111 (5B) or SNX-111, SNX-183 (5C), and cadmium to the bathing medium are indicated by hatch marks just above the ordinate;

Figures 6A and 6B are a binding curve showing the amount of radioiodinated OCT MVIIA (SNX-111) bound to rat synaptosomal membranes, as a function of OCT MVIIA (SNX-111) concentration (6A), and the same data plotted as a Scatchard plot (6B);

7

Figures 7 shows reversibility of equilibrium binding of radioiodinated SNX-111 and SNX-183, and irreversibility of binding of radioiodinated SNX-124 to rat brain synaptosomal membranes;

5      Figures 8A and 8B show computer-fit competitive binding curves for omega-conopeptide binding to the OCT MVIIA (SNX-111) binding site in rat brain synaptosomes;

Figure 9A shows computer-fit competitive binding curves for omega-conopeptide binding to the OCT SVIB 10   (SNX-183) binding site in rat brain synaptosomes, and Figure 9B shows binding site competitive binding curves for binding to OCT MVIIC (SNX-230);

Figure 10 (A and B) shows SDS-PAGE autoradiograms of rat synaptosomal membranes having covalently bound 15   radioiodinated OCT MVIIA (SNX-111)(A) or covalently bound radioiodinated OCT SVIB (SNX-183)(B) added to the membranes in the presence (lanes c and f) or absence (lanes a,b and d,e) of non-radiolabeled OCT, where lanes a and d are control preparations in which no 20   cross-linking agent was added;

Figure 11 shows plots of competition by unlabeled SNX-111 and SNX-183 for binding of [$^{125}$I]-SNX-111 (11A) and [$^{125}$I]-SNX-183 (11B) to the 210 kilodalton band of polypeptides present in rat synaptosomal membranes;

25      Figure 12 shows autoradiograms of the distributions of [$^{125}$I]-SNX-111 (A,B,C,D) and [$^{125}$I]-SNX-183 (E,F,G,H) binding to coronal rat brain rostral (A,C,E,G) and caudal (B,D,F,H) sections labeled in the absence of excess nonradioactive SNX-III (A,B) 30   or SNX-183 (E,F) or in the presence of excess non-radioactive SNX-111 (C,D) or SNX-183 (G,H), in which "CA" indicates the CA$_3$ region of the hippocampus and "SN" indicates the substantia nigra;

Figure 13A shows the inhibition of 35   [$^3$H]norepinephrine release from rat hippocampal slices

8

as a function of OCT MVIIA (SNX-111) concentration
(solid bars are potassium stimulated and open bars are
basal values), and Figure 13B shows the dose-dependent
inhibition of potassium-stimulated release of
5    [³H]norepinephrine by OCT MVIIA (SNX-111; triangles),
and OCT SVIB (SNX-183; squares);

Figure 14 shows omega-conopeptide groupings;

Figure 15 shows the production of analgesia by a
submaximal intrathecal dose of morphine (0.5 μg)
10   administered alone (solid bars) and in the presence of
0.5 μg SNX-111 (hatched bars);

Figure 16 (A-C) shows effects of SNX-III(A), SNX-
185(B) and SNX-231(C) on flinch response in rat
formalin tests;

15   Figure 17 shows log dose response curves for
effects of SNX-III, SNX-185 and SNX-231 on phase 1(A)
and phase 2(B) of the formalin test;

Figure 18 shows the effect of treatment with
saline (open circles), 0.3 μg SNX-111 (closed circles),
20   1 μg (closed squares), or 3 μg (closed triangles) SNX-
111 on response of neuropathic rats to a wire hair as
percent maximum effect; and

Figure 19 shows a histogram of the peak responses
shown in Figure 18, and comparison to peak response to
25   neuropathic rats treated with 10 μg morphine.


## Detailed Description of the Invention

### I.   Omega-conopeptides

Omega-conopeptides are components of peptide
30   toxins produced by marine snails of the genus Conus,
and which act as calcium channel blockers (Gray).
About 500 species of cone snails in the Conus genus
have been identified, and a variety of omega-
conopeptides from several of these species have been
35   isolated.   The primary sequences of eight natural

9

omega-conopeptides are shown in Figure 1, where SNX-231
is an alternative form of MVIIC/SNX-230. Conventional
letter initials are used for the amino acid residues,
and X represents 4-hydroxyproline, also abbreviated

5   4Hyp. All of the peptides shown in the figure are
amidated at their C-termini.

The peptides shown in Figure 1 are identified by
names which are commonly associated with either the
naturally occurring peptide (single letter followed by

10  a Roman numeral followed by a single letter), and by a
synthetic designation (SNX-plus numeral). Either or
both of these designations will be used interchangeably
throughout the specification. For example, the peptide
whose sequence is designated MVIIA/SNX-111 will be

15  referred to herein as OCT MVIIA, or alternatively, SNX-
111, the latter to signify that the compound is
synthetic in origin. Synthetic and naturally occurring
peptides having the same sequence behave substantially
identically in the assays and methods of treatment of

20  the invention. The OCT MVIIA (SNX-111) and OCT GVIA
(SNX-124) peptides also have the common names CmTx and
CgTx, respectively. All of the omega-conopeptides have
three disulfide linkages connecting cysteine residues
1 and 4, 2 and 5, and 3 and 6, as indicated for the

25  MVIIA peptide in Figure 2. Figure 2 shows analogs or
derivatives of natural OCT MVIIA, OCT TVIA, and OCT
SVIB peptides which have been synthesized and tested in
accordance with the invention. Standard single amino
acid code letters are used in the figure;

30  X=hydroxyproline; Nle=norleucine; NH$_2$ group at the C
terminus indicates that the peptide is C-terminal
amidated; G-OH indicates termination in an unmodified
glycine residue.

35

10

A. Preparation of Omega-conopeptides

This section describes the synthesis, by solid phase methods, of several naturally occurring omega conotoxin (OCT) peptides and additional omega-

5    conopeptides which are used in the present invention.

Omega-conopeptides, such as those shown in Figures 1 and 2, can be synthesized by conventional solid phase methods, such as have been described (Olivera). Briefly, N-alpha-protected amino acid anhydrides are

10   prepared in crystallized form and used for successive amino acid addition at the N-terminus. At each residue addition, the growing peptide (on a solid support) is acid treated to remove the N-alpha-protective group, washed several times to remove residual acid and to

15   promote accessibility of the peptide terminus to the reaction medium. The peptide is then reacted with an activated N-protected amino acid symmetrical anhydride, and the solid support is washed. At each residue-addition step, the amino acid addition reaction may be

20   repeated for a total of two or three separate addition reactions, to increase the percent of growing peptide molecules which are reacted. Typically, 1-2 reaction cycles are used for the first twelve residue additions, and 2-3 reaction cycles for the remaining residues.

25       After completing the growing peptide chains, the protected peptide resin is treated with liquid hydrofluoric acid to deblock and release the peptides from the support. For preparing an amidated peptide, the resin support used in the synthesis is selected to

30   supply a C-terminal amide, after peptide cleavage from the resin. The three disulfide linkages in the peptides may be formed by air oxidation in the presence of dithiothreitol (DTT) at room temperature or at 4°C over an extended reaction period. Alternatively, where

35   the correct or desired bridging cannot be achieved by

11

random oxidation, a chemically directed process may be used in which the bridges are formed sequentially, one bridge at a time. The following side-chain protecting groups could be used for each pair of cysteine

5   residues: 4-methylbenzyl, ethylcarbamoyl, and acetamidomethyl. These protecting groups constitute an orthogonal set in which any one kind of protecting group can be removed under conditions that do not affect the other two.

10       The strategy used in this method involves removing one kind of protecting group from a pair of cysteine residues, followed by oxidation to form the first disulfide bridge. A second kind of protecting group is then removed, again followed by oxidation to form the

15  second bridge. A third bridge, if needed, is formed in like manner.

         The peptide can be isolated by an initial separation by gel filtration, to remove peptide dimers and higher molecular weight polymers, and also to

20  remove undesired salts, such as guanidine hydrochloride, used in the oxidation reaction. The partially purified peptide is further purified by preparative HPLC chromatography, and the purity of the peptide confirmed by amino acid composition analysis.

25

B. In vitro Properties of Omega-conopeptides

1. Calcium-Channel Antagonist Activity

         Voltage-gated calcium channels are present in neurons, and in cardiac, smooth, and skeletal muscle

30  and other excitable cells, and are known to play a variety of roles in membrane excitability, muscle contraction, and cellular secretion, such as in synaptic transmission (McCleskey). In neuronal cells, voltage-gated calcium channels have been classified by

35  their electrophysiological as well as by their

12

biochemical    (binding)    properties.

Electrophysiologically, these channels can be classified either as Low-voltage-activated (LVA) or High-voltage-activated (HVA).    HVA channels are
5   currently known to comprise at least three groups of channels, known as L-, N- and P-type channels (Nowycky, Sher).  These channels can be distinguished electrophysiologically as well as biochemically on the basis  of  their  pharmacology  and  ligand  binding
10  properties.  ' Thus,    dihydropyridines, diphenylalkylamines and piperidines bind to the alpha$_1$ subunit of the L-type calcium channel and block a proportion of HVA calcium currents in neuronal tissue, which are termed L-type calcium currents.

15        Omega conotoxins also block a proportion of HVA calcium currents in neuronal tissue, and, in the presence of a maximally inhibitory quantity of dihydropyridine compound, substantially inhibit the remaining HVA currents in neuronal cells.   These
20  calcium currents are generally identified as N-type calcium currents.   Based on their pharmacological sensitivity, such currents are also termed "omega" currents by some authors (Sher).

Omega conotoxins bind to a specific population of
25  binding sites, present mainly in neuronal tissue. Dihydropyridines and other L-type channel blockers do not displace omega conotoxin binding, nor do omega conotoxins displace binding of ligands to L-type calcium channels.  Unlike L-type calcium channels, N-
30  type or omega channels are found predominantly, although not exclusively, in nervous tissue (Sher).

One suitable system for testing inhibition (blockage) of N-type or omega HVA neuronal calcium channels is an isolated cell system, such as the mouse
35  neuroblastoma cell line, strain N1E115 or the human

13

neuroblastoma cell line IMR32.  Membrane currents are
conveniently measured with the whole cell configuration
of the patch clamp method, according to the procedure
detailed in Example 1.   Briefly, a voltage clamp
5   protocol was performed in which the cell potential was
stepped from the holding potential of about -100 mV to
test potentials that ranged from -60 mV to +20 mV, and
the cell was held at the holding potential for 5
seconds between pulses.

10        Figure 3 shows a typical inward calcium current
elicited by a voltage step from -80 mV to -20 mV in the
absence of OCT.   In this, and most of the recordings
shown, barium (Ba) replaced calcium (Ca) as the
charge-carrier through the calcium channels in order to
15   increase the signal (McCleskey).   According to the
procedure   described   in   Example   1,   an   N1E115
neuroblastoma cell was bathed in saline with sodium
replaced by N-methyl-D-glucamine (NMDG), and 10 mM Ba
instead of 2 mM Ca.  These substitutions reduced the
20   sodium current that would otherwise have contaminated
the calcium current record, and increased the calcium
current above what it would have been with only 2 mM Ca
in the bath.   Potassium currents were blocked by
tetraethylammonium (TEA) in the bath and cesium (Cs) in
25   the pipet solution.

As   seen   from   Figure   3,   curve A,   the calcium
current activates quickly (within about 20 ms) and
inactivates with a time constant of 30 to 40 ms.  The
calcium current is measured by the amplitude of the
30   peak inward current elicited by the depolarization
peak, and has a measured value of about -1200 pA.  The
cell in Figure 3 (curve A) was also exposed to 1μM
nifedipine, a dihydropyridine, which is expected to
effectively block L-type calcium channels in the

14

neuroblastoma cells, and no effect on the measured
calcium current was observed. The calcium current
observed is thus not dihydropyridine-sensitive.

The responses of voltage-gated calcium currents to
increasing concentrations of OCTs MVIIA (SNX-111) and
GVIA (SNX-124) are shown in Figure 4. The $IC_{50}$
concentration, at which 50% inhibition of calcium
current is produced, is determined from the voltage-
gated current amplitudes, plotted as a function of
omega-conopeptide concentration. The calculated $IC_{50}$
is about 10 nM for GVIA and 100 nM for MVIIA,
indicative of high inhibitory peptide activity. The
$IC_{50}$ concentration for these and omega-conopeptides
SVIA (SNX-157) and SVIB (SNX-183) are given in Table 1
below.

Table 1
Inhibition of calcium currents in N1E-115
neuroblastoma cells

| Compound | $IC_{50}$ |
|---|---|
| GVIA (SNX-124) | 10nM |
| MVIIA (SNX-111) | 100nM |
| SVIB (SNX-183) | > 1$\mu$M |
| SVIA (SNX-157) | >20$\mu$M |

Calcium currents were also measured in human
neuroblastoma IMR32 cells, using techniques described
above and in Example 1. Voltage-gated calcium currents
were elicited by holding the cell(s) at -70 mV and
administering a step-voltage to -10 mV. Current
tracings from IMR-32 cells bathed in control medium
(lower curve) and in medium containing 150 nM SNX-111
(upper curve) are shown in Figure 5A. The amplitude of
the current is shown on the abscissa. The peak inward

15

current is shown as the difference between the resting potential shown at the far left side of the figure and the lowest point of the curve, just adjacent to the resting value.   In this experiment attenuation of

5   voltage-gated calcium current is apparent in the presence of SNX-111 (upper curve), as shown by the decreased amplitude of the peak inward current.

Figures 5B and 5C show cumulative data from many consecutive currents, elicited at 15 second intervals

10  as described above, in IMR-32 cells.   In these plots, peak inward current recorded from each stimulus is recorded sequentially as a single data point.   In the experiment illustrated in Figure 5B, addition of SNX-111 to the bathing medium resulted in decreased peak

15  inward currents; restoration of substantially normal calcium currents was achieved after washing of the compound from the cell chamber, shown on the right side of the figure.   Figure 5C shows the effects of 150 nM SNX-111 and SNX-183 added sequentially to a single cell

20  preparation.  Both compounds resulted in attenuation of peak inward current; though recovery following SNX-183 exposure was not observed.  Addition of cadmium (Cd) to the medium resulted in blockade of all remaining voltage-gated calcium currents in this preparation.

25  Test peptides which are inhibitory for neuronal cell calcium currents can be further tested in non-neuronal cells, to confirm that the peptide activity in blocking calcium currents is specific to neuronal cells.   A variety of muscle cell types which are

30  refractory to calcium-current inhibition by OCTs, such as vertebrate embryo heart and skeletal muscle cells, are suitable.   Cell current measurements are made substantially as outlined above and detailed in Example 1.  OCT MVIIA, for example, has been reported to block

35  voltage-gated calcium channels in a variety of neuronal

16

cells, including dorsal root ganglion (DRG) neurons
(McCleskey). This blockage or inhibition of calcium
channel currents has been reported to be neuron-
specific, since calcium current inhibition by the
5   peptide was not observed in cardiac, smooth, and
skeletal muscles.

2.   Specific, High Affinity Binding to OCT Receptors
     Omega-conopeptides have been shown, in accordance
10  with the invention, to bind with high affinity to
specific binding site(s) in neuronal cells. In
accordance with the selectivity of the compound, the
binding affinity can be characterized either by the
binding constant of the compound for the MVIIA (SNX-
15  111) binding site, also referred to as "site 1" herein,
or the binding constant of the compound for the SVIB
(SNX-183) or the MVIIC (SNX-230) binding site, also
referred to as "site 2" herein. Evidence for the
existence of at least two distinct OCT binding sites is
20  summarized below. In some cases, when specific binding
to one site is preferred, it will be useful to
characterize omega-conopeptides according to the ratio
of their binding constants measured for binding to
neuronal-cell MVIIA (SNX-111) binding site 1 and SVIB
25  (SNX-183) or MVIIC (SNX-230) binding site 2.
     Binding to the OCT MVIIA binding site in neuronal
tissue can be demonstrated in a variety of cell types
and synaptosomal cell fractions. One preferred
synaptosomal fraction is a mammalian brain synaptosomal
30  membrane preparation, such as the rat brain synaptosome
preparation described in Example 2. The binding
constant of a compound for the MVIIA binding site is
typically determined by competitive displacement of
radiolabeled OCT MVIIA (SNX-111) from the synaptosomal
35  preparation, as follows.

17

The binding constant $K_d$ of the MVIIA (SNX-111) peptide for the synaptosomal membranes is determined by a saturation binding method in which increasing quantities of radiolabeled peptide are added to the
5    synaptosomal membranes, and the amount of labeled material bound at each concentration is determined (Example 3A). The plot of bound peptide as a function of concentration is then used to calculate a $B_{max}$, the concentration of binding sites on the synaptosomes, and
10   $K_d$ following standard methods. In particular, the $K_d$ value is the calculated concentration of peptide needed to half saturate the synaptosomal specific binding sites. Figure 6A shows the specific binding of radiolabeled OCT MVIIA (SNX-111) to rat brain
15   synaptosomes, plotted as a function of omega-conopeptide concentration, and Figure 6B, the same data in Scatchard plot form. From the slope of the Scatchard plot line, a $K_d$ binding value of 10 pM is obtained. Similarly $K_d$'s were determined for binding
20   of radiolabelled SVIB (SNX-183) to binding sites in synaptosomal membranes.

Reversibility of binding is a characteristic of ligands which, under equilibrium conditions, freely associate with and dissociate from their respective
25   binding sites. Reversibility of binding of a specific compound is demonstrated by the labelled compound's ability to be displaced by unlabelled compound, after equilibrium binding of the labelled compound has been achieved. For example, dissociability of binding of a
30   labelled compound can be determined as detailed in Example 3B, where a synaptosomal preparation was incubated with labelled compound for a time period sufficient to produce a stable level of binding, then excess unlabelled compound was added to the
35   preparation. The preparation was then assayed for

18

bound labelled compounds at various timepoints after addition of unlabelled compound.

If the labelled compound binds reversibly to the preparation, a reduction of labelled binding, to essentially non-specific binding levels, should be observed over time. Figure 7 shows a plot of the dissociation kinetics of labelled SNX-111, SNX-183 and SNX-124. In contrast to SNX-111 binding, labelled OCT GVIA (SNX-124) does not dissociate from synaptosomal membranes over the course of an hour and has a calculated $t_{1/2}$ of 19 hours. SNX-124 binding can therefore be said to be essentially irreversible, while SNX-111 and SNX-183 bind reversibly to their respective binding sites.

To determine the binding constant of a test compound for an OCT binding site, the test compound is added, at increasing concentrations, to the synaptosome preparation in the presence of a standard concentration of a radiolabeled OCT which exhibits reversible binding, such as OCT MVIIA (SNX-111). The synaptosomal material is then rapidly filtered, washed and assayed for bound radiolabel. The binding constant ($K_i$) of the test compound is determined from computer-fit competitive binding curves, such as shown in Figures 8A and 8B for MVIIA (SNX-111) peptide, to determine first the $IC_{50}$ value of the compound, i.e., the concentration which gives 50% displacement of labeled MVIIA peptide.

A $K_i$ is determined according to standard methods from the $K_d$ value of OCT MVIIA and the $IC_{50}$ value of the compound, as detailed in Example 3. A relative potency value can also be calculated from this information (Example 3). Like the $K_i$ value, this value allows comparisons between assays performed under slightly differing conditions or at different times. Calculated $IC_{50}$ values for a number of omega-conopeptides for

19

binding of OCT MVIIA (SNX-111) are given in Table 2.
The compounds are arranged in order of increasing $IC_{50}$
values.

5

| Table 2<br>Competition of $^{125}$I-MVIIA (SNX-111)<br>Binding by OTC Peptides | |
|---|---|
| | $IC_{50}$ (nM) |
| SNX-207 | .007 |
| SNX-194 | .008 |
| SNX-195 | .009 |
| MVIIA (SNX-111) | .013 |
| SNX-190 | .021 |
| SNX-200 | .039 |
| SNX-201 | .046 |
| SNX-202 | .046 |
| SNX-193 | .070 |
| MVIIC (SNX-230) | .32 |
| MVIIB (SNX-159) | .101 |
| GVIA (SNX-124) | .134 |
| SNX-198 | .160 |
| SNX-191 | .165 |
| TVIA (SNX-185) | .228 |
| SNX-196 | .426 |
| RVIA (SNX-182) | .893 |
| SVIB (SNX-183) | 1.5 |
| GVIIA (SNX-178) | 3.70 |
| SNX-197 | 11.3 |
| SVIA (SNX-157) | 1460. |

10

15

20

25

30

Similarly, $IC_{50}$ and $K_i$ values for compound binding
to the SVIB (SNX-183) binding site can be calculated,

20

as above, by determining the $K_d$ of labeled OCT SVIB
(SNX-183) or OCT MVIIC (SNX-230) binding to a
synaptosome preparation, then using competitive
displacement of the labeled compound by the test
5    compound, to determine the $IC_{50}$ and $K_i$ or relative
potency values of the test compound. Figures 9A and 9B
show computer-fit competitive binding curves for
several omega-conopeptides whose binding to the SVIB
(SNX-183) and MVIIC (SNX-230) binding sites was
10   examined. From these curves, $IC_{50}$ values were
determined as above.

Table 3 lists the relative potencies for binding
of various omega-conopeptides to the site 1 and site 2
binding sites, and shows the ratio of Ki values
15   determined for binding of each compound to the sites.

Table 3

Selectivity of Conopeptides for Site 1 and Site 2

| Compound | Ki (nM) for competition[a] with: | | Selectivity[b] for: | |
|---|---|---|---|---|
| | [$^{125}$I]-SNX-111 | [$^{125}$I]-SNX-230 | site 1 | site 2 |
| SNX-111 | 0.002 | 150 | 75,000 : 1 | |
| [$^{125}$I]-SNX-111 | 0.002 | 320 | 39,400 : 1 | |
| SNX-183 | 0.43 | 6 | 14 : 1 | |
| [$^{125}$I]SNX-183 | 1.25 | 1.5 | 1.2 : 1 | |
| SNX-230 | 0.20 | 0.03 | 1 : 7 | |
| [$^{125}$I]-SNX-230 (SNX-260) | 0.49 | 0.02 | 1 : 25 | |

[a]Ki values were derived from analysis of
competitive binding performed as described in
Figure 1.

[b]Selectivity is expressed as the ratio of the Ki
value determined for competition with [$^{125}$I]-SNX-
230 binding divided by the Ki value for
competition with [$^{125}$I]-SNX-111 binding.

21

The identity of the MVIIA and SVIB binding sites in neuronal-cell membranes was examined by binding radiolabeled OCT MVIIA to synaptosomes, and crosslinking the peptide to the neuronal membranes, as

5    detailed in Example 4.  The labeled membranes were solubilized with sodium dodecyl sulfate (SDS), fractionated by polyacrylamide gel electrophoresis (PAGE), and examined by autoradiography for labeled polypeptide bands.  In one case, the membranes were

10    incubated with labeled peptide in the presence of excess unlabeled OCT MVIIA.  A similar binding study was carried out with labeled OCT SVIB.

Evidence that the two receptor sites identified by SNX-111 and SNX-183 are distinct was obtained from

15    affinity crosslinking studies in which $[^{125}I]$-SNX-111 and $[^{125}I]$-SNX-183 were chemically crosslinked to rat brain synaptosomal membrane preparations and then subjected to SDS-PAGE followed by autoradiography (Fig. 10).  $[^{125}I]$-SNX-111 specifically labelled a protein of

20    $M_r$ 210-220 kDa, as indicated in the drawing. Unlabelled SNX-111 inhibited labeling of this protein with radioactive SNX-111 with an $IC_{50}$ of 30 pM, in good agreement with the $IC_{50}$ for site 1 determined by binding assays (Fig. 8).  Labeling of this 210 kDa protein band

25    by $[^{125}I]$-SNX-111 was also inhibited by SNX-183 but with lower affinity ($IC_{50}$ = 300pM).  Similar experiments with $[^{125}I]$-SNX-183 revealed that in addition to the expected labeled band at 210-220 KDa, three additional bands at 172, 150 and 138kDa appear to be specifically labeled

30    (Fig. 10B).

Analysis of the inhibition of incorporation of $[^{125}I]$-SNX-183 in the 210kDa band by SNX-111 provides evidence for the presence of two distinct polypeptides of $M_r$ 210kDa corresponding to site 1 and site 2 (Fig.

35    11).  SNX-111 displaced $[^{125}I]$-SNX-183 from the 210kDa

22

polypeptide in a biphasic manner with $IC_{50}$ values of 6 pM and 65 nM. At low concentrations, SNX-111 effectively competed against $[^{125}I]$-SNX-183 for binding to site 1, while the binding of $[^{125}I]$-SNX-183 to site 2 was competitively displaced by SNX-111 only at much higher concentrations.

Inhibition of incorporation of $[^{125}I]$-SNX-183 into the 210kDa band by SNX-183 is consistent with the ability of this compound to bind to both site 1 and site 2, but, as shown above, with much lower affinity than MVIIA (SNX-111) or TVIA (SNX-185) at site 1. The displacement curve displacement of MVIIA binding by SNX-183 is characteristically shallow, with an $IC_{50}$ of 360 pM (Figure 11A). Taken together with the differential rank orders of binding affinities for omega-conopeptides at the two binding sites, as measured by displacement, these crosslinking experiments support the idea that the conopeptide binding components of site 1 and site 2 are distinct molecular entities, both with $M_r$ 210-220 kDa.

3. Localization of Binding of Omega-conopeptides in Nervous Tissue

The omega-conopeptide binding sites described above are distributed differentially throughout the nervous system. The regional distribution of the binding sites and their relative affinities for the two conopeptides SNX-111 and SNX-183 in rat brain sections were determined by autoradiography of brain sections exposed to the radiolabelled compounds, detailed in Example 5. The results presented in Fig. 12 show that the distribution of binding of $[^{125}I]$-SNX-111 is highly localized (A, B) and that non-specific binding is virtually non-existent (C, D). The pattern of binding is similar to that reported using $[^{125}I]$-GVIA

23

preparations (Takemura). Comparison of the specific binding of $[^{125}I]$-SNX-111 and $[^{125}I]$-SNX-183 revealed overlapping but differential distribution of binding sites (E, F). Both ligands labeled the cortex, CA1,
5 dentate gyrus and caudate-putamen. In these regions, binding of $[^{125}I]$-SNX-183 was unaffected by concentrations of SNX-111 which caused complete displacement of $[^{125}I]$-SNX-111 labeling (not shown), suggesting colocalization of sites 1 and 2. Greater
10 abundance of site 2 in thalamic ventromedial lateral lobe and medial geniculate was revealed by the high density of binding of $[^{125}I]$-SNX-183 in these nuclei. In contrast, globus pallidus, $CA_3$ and substantia nigra were labeled only by $[^{125}I]$-SNX-111, indicating a
15 preponderance of site 1 in these regions. The complete absence of $[^{125}I]$-SNX-183 binding in the substantia nigra suggests a third, distinct binding site recognized only by $[^{125}I]$-SNX-111, and by implication, a novel calcium channel subtype.
20 Computer-aided densitometric analysis of the displacement of $[^{125}I]$-SNX-111 by SNX-183 in the different brain regions showed that the labeling of cortex and hippocampus by $[^{125}I]$-SNX-111 could be inhibited by lower concentrations of SNX-183 ($IC_{50}$ 100
25 nM), whereas higher concentrations of SNX-183 ($IC_{50}$ 300 nM) were needed to displace $[^{125}I]$-SNX-111 from the substantia nigra. Since a number of nuclei that are known to be rich in synapses and thus likely to contain a high density of presynaptic calcium channels were not
30 labeled by either ligand, the two conopeptides can distinguish four different subtypes of neuronal binding sites, as summarized in Table 4. The four subtypes are, those sensitive to both SNX-111 and SNX-183 (site 1), those sensitive to SNX-183 only, (site 2), those
35 recognized by SNX-111 only and others recognized by

24

neither conopeptide.

## Table 4
### Four classes of OCT binding site calcium channels

| Site | Binds SNX-111 | Binds SNX-183 | Examples |
|------|-----|-----|----------|
| 1 | + | + | c o r t e x , hippocampal CA1, CA3, thalamic nuclei, spinal cord (laminae I+II only) |
| 2 | – | + | c o r t e x , hippocampal CA1, CA3, thalamic nuclei |
| 3 | – | – | midbrain nuclei, spinal grey matter (except laminae I + I I ), n e u r o m u s c u l a r junction |
| 4 | + | – | substantia nigra, hippocampal CA2 |

### 4. Selective Inhibition of Neurotransmitter Release

Omega-conopeptides inhibit neurotransmitter release in various regions of the nervous system. As shown below, such inhibition varies according to the neurotransmitter, the omega-conopeptide, and the region studied. Neurotransmitters which can be measured, in accordance with various aspects of the invention, include, but are not limited to dopamine, norepinephrine, acetylcholine, GABA, glutamate, and a number of peptide neurotransmitters, such as substance P (McGeer).

Quantitation of release and inhibition thereof is determined by sensitive detection methods, also known in the art, including direct detection of release of

25

endogenous stores by HPLC or specific radioimmunoassay
(RIA), and detection of release of pre-loaded, labeled
compound. Alternatively, or in addition, detection of
release may be achieved using a number of indirect
5    assays, exemplified by the electrophysiological studies
described above, in which whole tissue response to
electrical or chemical stimulation is measured.

        Inhibition of release of the neurotransmitter
norepinephrine from neuronal cells can be assayed in
10   mammalian brain hippocampal slices by standard methods,
such as detailed in Example 6A. Briefly, hippocampal
slices are distributed to individual wells of a
microtiter plate, and incubated with radiolabeled
norepinephrine under conditions favoring cellular
15   uptake of the transmitter. The cells are washed with
a low-potassium medium, then bathed for 15 minutes in
a high-potassium stimulation medium, in the presence of
selected concentrations of a test compound. After
removal of the stimulation buffer, radioactivity
20   remaining in each slice is determined.

        Figure 13A shows concentration dependence of
inhibition of norepinephrine release from hippocampal
slices, as detailed in Example 8A. Basal (open bars)
and potassium-stimulated (solid bars) release is shown
25   in the presence of varying concentrations of SNX-111,
as indicated.

        Fig. 13B shows the effects of the three peptides
SNX-111, SNX-183 and SNX-230 on the release of
norepinephrine evoked by potassium depolarization.
30   SNX-111 inhibits release with high potency ($IC_{50} \approx 1$
nM) but only partially (approx. 60%). SNX-183 is much
less potent ($IC_{50} \approx 180$ nM) but the inhibition is
substantially 100%. SNX-230 also inhibits release
100%, but in a biphasic manner, inhibiting

26

approximately 50% with high potency ($IC_{50}$ = 0.02 nM)
and 50% with much lower potency ($IC_{50}$ = 65 nM).   In
agreement with the binding studies discussed above,
these results imply that such norepinephrine release is
5    mediated   by   at   least   two   distinct   subtypes   of
presynaptic calcium channels, one of which corresponds
to the site 1 receptor identified by high affinity for
SNX-111 and the other to the site 2 receptor recognized
preferentially by SNX-230.

10    The $IC_{50}$ values given in Table 5 for a variety of
omega-conopeptides which have been examined by this
method represent the average $IC_{50}$ values calculated
from thin (200 $\mu$) and thick (400 $\mu$) hippocampal slices.
The three lowest $IC_{50}$ values, between 0.8 and 2.4 nM,
15    correspond to omega-conopeptides which are most potent
in this assay.

| Table 5 | |
|---|---|
| Inhibition of Norepinephrine Release by Omega-conopeptides | |
| omega--conopeptides | $IC_{50}$ (nM) |
| GVIA (SNX-124) | 0.8 |
| MVIIA (SNX-111) | 1.5 |
| TVIA (SNX-185) | 2.4 |
| SNX-201 | 11 |
| SNX-195 | 11 |
| SNX-202 | 29 |
| SVIB (SNX-183) | 200 |
| SNX-191 | >100 |
| SVIA (SNX-157) | >4500 |

The  selective  effect  of  omega-conopeptides  in
inhibiting neurotransmitter release from specific types

27

of nerve terminals is demonstrated by the markedly different responses of other neurotransmitter systems, when exposed to omega-conopeptides. When tested in a synaptosomal preparation prepared from the neuronal

5    rich electric organ of electric eel (<u>Ommata dyscopyge</u>), a different rank order of potency was observed (Table 6).

## Table 6

10    Inhibition of Release of ATP from Electric Organ Synaptosomes

| Compound | $IC_{50}$ |
|----------|-----------|
| SNX-111 | 42 |
| SNX-195 | 84 |
| SNX-183 | 1,700 |
| SNX-185 | >6,000 |
| SNX-157 | >6,000 |

Similarly, inhibition of release of amino acid

20    neurotransmitters GABA and glutamate from rat neuronal tissue did not parallel either binding potency at the MVIIA site 1, the SVIB site 2 or inhibition of norepinephrine release (Table 7).

25                      **Table 7**

Inhibition of release of GABA and Glutamate from rat Synaptosomes

| Compound | $IC_{50}$ (GABA) | $IC_{50}$ (Glutamate) |
|----------|-----------------|----------------------|
| SNX-185 | 100 nM | >100 nM |
| SNX-183 | 200 nM | 200 nM |
| SNX-111 | >200 nM | >200 nM |

Effects of omega-conopeptides were also compared to those of OCT GVIA and amiodipine, an L-channel

35    blocker, on potassium-stimulated release of dopamine

28

and acetylcholine from slices of rat brain (striatal
region) as described in Example 6 (C,D). Briefly, in
these experiments, striatal slices from rat brain were
preloaded with radiolabelled dopamine or choline, then

5   perfused for 45 minutes with bathing media. Slices
were subjected to an S1 stimulus; consisting of
addition of 15 mM potassium chloride to the bathing
medium for 1 minute. Total outflow of radiolabeled
neurotransmitter in response to S1 was measured.

10  Slices were then washed, exposed to test compound for
20 minutes, then subjected to an S2 stimulus, as above.
Comparison of outflow of neurotransmitter in response
to S2 to outflow in response to S1 is a measure of drug
effects on the system. Results are given as percent

15  inhibition of release in
Tables 8 and 9.

### Table 8

Effect of Omega-conopeptides and amiodipine on [$^3$H]
dopamine release from striatal slices

| Compound | Concentration | % inhibition |
|----------|---------------|--------------|
| GVIA | 1 nM | 5 |
|  | 10 nM | 52 |
| MVIIA | 1 nM | 6 |
|  | 10 nM | 49 |
| Amiodipine | 1000 nM | 0 |

### Table 9

Effect of Omega-conopeptides and amiodipine on [$^3$H]
acetylcholine release from striatal slices

| Compound | Concentration | % inhibition |
|----------|---------------|--------------|
| GVIA | 3 nM | 50 |
| MVIIA | 5.5 nM | 50 |
| Amiodipine | 1000 nM | 0 |

29

Further means of measuring inhibition of neuronal transmitter release are isolated tissue assays, such as atrial strip, aorta, vas deferens and guinea pig ileum assays, in which the response to a stimulus, usually an electrical stimulus, is correlated to the amount of neurotransmitter released from neurons innervating the tissue (Kenakin). In the guinea pig ileum, inhibition of electrically stimulated contractions is correlated with inhibition of acetylcholine release, as demonstrated by the ability of cholinergic agonists to overcome such inhibition. Example 7E describes the preparation and assay in detail. Table 10 shows the $IC_{50}$ values for various omega-conopeptides on contraction of guinea pig ileum in response to electrical stimulation.

## Table 10

Effects of conopeptides on electrically stimulated
contraction of Guinea pig ileum

| Compound | $IC_{50}$ (nM) |
|---|---|
| SNX-111 | 13 |
| SNX-185 | 29 |
| SNX-183 | 91 |
| SNX-157 | >100 |

## II. Treatment of Pain

In accordance with one aspect of the invention, it has been discovered that omega-conopeptides TVIA (SNX-185) or MVIIA (SNX-111), or derivatives thereof which are effective (a) to inhibit voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) to bind to omega conopeptide MVIIA binding sites present in neuronal tissue, are effective to produce

30

analgesia and to enhance the analgesic effect of an
opiate compound.

A.    Omega-conopeptides

5          Omega-conopeptides useful in the treatment of pain
have been found, in accordance with the invention, to
conform to certain physical and chemical constraints,
as described below.    Generally, omega-conopeptides
useful in the treatment methods are those which are 25-
10    35 amino acids in length and which have three disulfide
bonds at specified positions along their length.
           Based on a sequence homology analysis of the pep-
tides whose full sequences are known (Figure 1), the
naturally occurring active omega-conopeptides were
15    grouped into distinct groups I and II, each with
internal homologies distinct to that group, as can be
appreciated from Figure 14.    Group I includes active
omega-conopeptides MVIIA (SNX-111) and MVIIB (SNX-159)
which possesses a binding constant to the MVIIA site
20    within the range of compounds showing activity in
treating pain.    Group II includes TVIA (SNX-185), SNX-
207 and SNX-236.    A third group includes inactive
peptides SNX-231, and SVIA (SNX-157) and omega-
conopeptides whose binding activities for the MVIIA
25    site on neuronal membranes and/or activity in
norepinephrine inhibition are outside the range of
active compounds.
           The three groups of omega-conopeptides are
arranged in Figure 14 with their six Cys residues
30    aligned, which places these residues at positions 1, 8,
15, 16, 20, and 28.    To make this alignment, gaps were
introduced at the positions shown in the three groups.
In the analysis below, these gaps retain the assigned
number shown in Figure 14, even though they represent
35    amino acid deletions in the respective groups of active

31

omega-conopeptides.

Sequence variation in the peptides, based on primary structure alone, was analyzed by adopting the following constraints:

5          1. The peptides in both active groups (I and II) include the Cys residues at position 1, 8, 15, 16, 20, and 28. Other Cys residues could be substituted at the positions indicated below only if they are selectively protected during oxidation of the peptide to form the

10    three disulfide linkages.

2. The peptides in the active groups include three disulfide linkages connecting the Cys residues at positions 1 and 16, 8 and 20, and 15 and 28. As described above, the disulfide bridges are formed by

15    air oxidation of the full sequence peptide in the presence of DTT. The ability of the peptide to form the three desired disulfide linkages would therefore require that the peptide, prior to disulfide bridging, be able to adopt a conformation which allows the three

20    selected linkages, with or without the Cys protecting-group strategy discussed above. This constraint would thus exclude amino acid variations which prevent or otherwise hinder the formation of the three selected bridges.

25          Constraints 1 and 2 preserve the basic conformation of the omega-conopeptides imposed by the three disulfide bridges.

3. Within Group I, the amino acid variations which occur at the six non-conserved residues are

30    allowed, including peptides in which the carboxy terminus is amidated or has a free acid form. That is, the first group compound derivatives include the peptide structures having the form: SEQ ID NO: 22-$X_1$-SEQ ID NO: 23-$X_2$-SEQ ID NO: 25-$X_3X_4$-SEQ ID NO: 24-$X_5$-SEQ

35    ID NO: 25-$X_6$-SEQ ID NO: 26-t, where $X_1$=K or S; $X_2$=S or

32

H; $X_3$=L or T; $X_4$=M or S; $X_5$= N or a deletion; $X_6$=S or
deletion, and t= a carboxy or amidated carboxyterminal
group, and where SEQ ID NO: 22 is C K G K G A; SEQ ID
NO: 23 is C; SEQ ID NO: 25 is R; SEQ ID NO: 24 is Y D
5    C C T G S C; and SEQ ID NO: 26 is G K C.

     4.  Within Group II, the amino acid variations
which occur at the eight non-conserved residues are
allowed, including peptides in which the carboxy
terminus is amidated or has a free acid form.  Thus,
10   the second group compound derivatives include the
peptide structures having the form: SEQ ID NO: 27-
$X_1X_2X_3$-SEQ ID NO: 28-$X_4$-SEQ ID NO: 29-t, where $X_1$=X or R;
$X_2$=T or L; $X_3$= S or M, $X_4$= X or P; and t= a carboxy or
amidated carboxyterminal group, and where SEQ ID NO: 27
15   is C L S X G S S C S; SEQ ID NO: 28 is Y N C C R S C N;
and SEQ ID NO: 29 is  Y S R K C R.

     5.  Considering both active groups together, amino
acid positions which are conserved in all active
species are preserved.  Thus, for example, the Cys
20   residues, the 5-position glycine, the 13-position
tyrosine, the 19-position serine, and the 26-position
lysine are all preserved.  Preferred OCT analogs or
derivatives may be selected by comparing, for purposes
of inter-sequence conservation and substitution, those
25   sequences known to be active.  For example, in the case
of the treatment of pain, omega-conopeptides MVIIA
(SNX-111) and TVIA (SNX-185) are known active
compounds.  Active derivatives are those peptides
having, in addition to the conserved cysteine residues
30   described above, a conserved glycine residue at
position 5,  conserved serine residues at positions 9,
19, and 24, and a conserved lysine residue at position
26.  Inter-sequence substitution of variable residues
is then preferable in the formation of active analogs.
35   For example, analog position 2 may be occupied by a

33

lysine or a leucine residue, and position 6 may be occupied by an alanine or a serine residue.

6. Considering both active groups together, there are amino acid positions which are likely to be variable within the range of active species. For example, the position 2 amino acid may be lysine or leucine, the position-3 amino acid may be glycine or serine, and the position 4 amino acid, hydroxyproline or arginine. In addition, if the two or more amino acids at a variant position are in a common substitution class, substitution within that class may be favorable. Standard substitution classes are the six classes based on common side chain properties and highest frequency of substitution in homologous proteins in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix (Dayhoff). These classes are Class I: Cys; Class II: Ser, Thr, Pro, Hyp, Ala, and Gly, representing small aliphatic side chains and OH-group side chains; Class III: Asn, Asp, Glu, and Gln, representing neutral and negatively charged side chains capable of forming hydrogen bonds; Class IV: His, Arg, and Lys, representing basic polar side chains; Class V: Ile, Val, and Leu, representing branched aliphatic side chains, and Met; and Class VI: Phe, Tyr, and Trp, representing aromatic side chains. In addition, each group may include related amino acid analogs, such as ornithine, homoarginine, N-methyl lysine, dimethyl lysine, or trimethyl-lysine in class IV, and a halogenated tyrosine in Group VI. Further, the classes may include both L and D stereoisomers, although L-amino acids are preferred for substitutions.

7. Considering the known inactive species, substitutions to amino acids which are present in inactive species, but not active ones, at any selected residue position, are not favored to preserve activity

34

in the active compounds. Thus, for example, although a 3-position serine is present in both active and inactive compounds, 4-position serine or threonine is present in inactive species only, and either substitution is thus disfavored.

The above amino acid selection rules 6-7 are intended as a guide for allowed amino acid substitutions within active omega-conopeptides. Once an amino acid substitution or modification is made, the peptide is further screened for the requisite calcium channel antagonist activity, and the requisite activities for inhibition of neurotransmitter release and binding to the appropriate OCT binding site of neuronal membranes, as described above.

Several of the amino acid substitutions or modifications to the omega-conopeptide illustrate the principles outlined above.

Omega-conopeptides which are selected on the basis of these criteria, discussed in detail below, are tested for ability to produce or enhance analgesic effects produced by sub-maximal doses of opioid compounds in a standard test of analgesia, such as the Rat Tail-Flick test, wherein analgesia is measured by a prolongation of reaction time to a noxious radiant heat stimulus.

B.  In vitro Properties of Analgesic Omega-conopeptides

1.  Calcium channel blocking activity.  Calcium channel blocking activity was measured electrophysiologically in neuronal (N1E-115 or IMR-32) cell lines, as described in Section II, above, and in detail in Example 1. Omega-conopeptides having calcium channel blocking activity are those which block calcium currents in such cell lines with potencies in the range observed for omega-conopeptides MVIIA and GVIA in N1E-

35

115 cells, or displaying the efficacy observed for omega-conopeptides MVIIA and SVIB in IMR-32 cells (Figure 5C).

2. <u>High Affinity Binding to OCT Binding Sites</u>. Methods for determination of binding affinity to OCT binding sites are discussed in Examples 2-4, below.

Experiments testing reversibility of binding of SNX-111, SNX-183 and SNX-124 revealed that SNX-111 and SNX-183 exhibited dissociation half-times of two and five minutes, respectively. In contrast, SNX-124 did not dissociate appreciably from its binding site(s), even 1 hour following addition of excess unlabelled compound.

Compounds were tested for their ability to displace binding of SNX-111 or SNX-183 from their respective binding sites. In displacing SNX-111, it was found that compounds which produce or enhance opioid anti-nociceptive activity, such as OCT MVIIA (SNX-111), and TVIA (SNX-185), have $IC_{50}$ values between about 15 and 300 pM, and $K_i$ values between about 1 and 100pM. In contrast inactive compound SNX-183 had an $IC_{50}$ of greater than 1000 pM for binding at the MVIIA site.

From the foregoing, it is seen that active compounds in accordance with the invention are characterized by a high binding affinity for MVIIA binding site 1. The binding affinity for these sites may be characterized as follows. In the first approach, the binding affinity of the compound for the MVIIA site, as estimated by $IC_{50}$ at the site, is compared directly with those of selected high affinity active compounds, such as SNX-111 and SNX-185. An active compound is one whose binding affinity is at least as high as and preferably within the range of binding affinities measured for such high affinity

36

OCT's. Secondly, the binding affinity of the test compound can be characterized by binding to SVIB binding site 2, as described above for binding to MVIIA binding site 1. Thirdly, the binding affinity of the
5 compound can be characterized by the ratio of binding constants or relative potencies of the compound for the MVIIA and SVIB sites, as just described. Here an active compound is one whose binding ratio is within the range for the selected active peptides, such as
10 MVIIA (SNX-111) and TVIA (SNX-185); i.e., the binding ratio is substantially within the range of the ratio observed for the omega-conopeptides MVIIA and TVIA.

A number of omega-conopeptide compounds which were tested gave $IC_{50}$ and $K_i$ values lower than or within the
15 ranges of those of omega-conopeptides MVIIA (SNX-111) and TVIA (SNX-185) for binding at the SNX-111 site, and these compounds should thus be considered candidates as anti-nociceptive enhancing compounds. However, some of these compounds, may not fulfill additional criteria
20 for anti-nociceptive compounds of the invention, as described herein.


3.  Localization of OCT binding in the central nervous system. Conopeptide SNX-111 binds to distinct
25 regions of the brain and spinal cord which are commonly associated with pain pathways (Figure 12). These include the periaquaductal grey (PAG) region of the brain and the dorsal horn of the spinal cord. The distribution of CgTx (GVIA) binding shown by Takemura
30 et al (1989) also shows localization of CgTx binding sites at a very high level in the dorsal horn of the spinal cord (1$^{st}$ and 2$^{nd}$ layers of Rexed) and, to a lesser degree, in the central grey region of the mesencephalon, which may correspond to the PAG;
35 however, some of these compounds may not fulfill

37

additional criteria for antinociceptive compounds of
the invention, as described herein.

    4.    Inhibition of neurotransmitter release.
Another requisite property of analgesic, anti-
nociceptive OCT compounds, in accordance with the
invention, is their ability to specifically inhibit
depolarization-evoked and calcium-dependent
neurotransmitter release from neurons. In the case of
anti-nociceptive omega-conopeptides, inhibition of
electrically stimulated release of acetylcholine at the
myenteric plexus of the guinea pig ileum (Example 6E)
is predictive of anti-nociceptive activity, as seen in
Table 10. Omega-conopeptides having anti-nociceptive
activity and/or anti-nociceptive enhancing activity
have $IC_{50}$'s in the range of those values observed for
active omega-conopeptides MVIIA (SNX-111) and TVIA
(SNX-185), or less than approximately 50 nM, as shown
in this assay.


    5.    In vivo measurements of analgesia. Analgesia
is conveniently measured in one or more of a number of
animal models, in which an animal's response to a given
pain stimulus is measured. One such model is the Rat
Tail-Flick test, described in Example 7. Briefly, in
this test, a rat is positioned such that its tail is
exposed to a standard heat source, and the time that
the animal voluntarily endures the heat, prior to
moving its tail, is recorded. Analgesics, particularly
opioid analgesics, prolong this time.
    Shown in Figure 15 are the results of experiments
in which the effects of a sub-maximal dose of morphine
were compared to those of the combination of a sub-
maximal dose of morphine and a 0.5 μg (intrathecal)
dose of SNX-185 in the Rat Tail-Flick Test. Animals
were tested at various time points following injection,

38

as indicated, and latency of tail-flick recorded. In addition, for each animal, the maximal latency response was recorded, and the mean of such maximal responses calculated as the maximal percent effect (MPE).

5   Intrathecal administration of SNX-185 enhanced the effects of a sub-maximal dose of morphine (Figure 15) in this assay at all time points, and significantly at 45 min. after administration of compound. By the term "submaximal dose" is meant a dose of morphine or other

10   opiate which is insufficient to induce maximal latency of response in the tail-flick assay, measured as Percent Effect, the calculation of which is described in Example 7.

Table 11 shows the effects of SNX-185 given alone

15   or in combination with varying doses of morphine, shown on the right side of the table. SNX-185 showed a significant analgesic effect when given alone at a dose of 0.5 μg, in at least one of the trials. When given in conjunction with a low dose (0.05 μg) of morphine, 0.5

20   μg SNX-185 yielded a significant enhancement of the effect of morphine alone.

In separate experiments (Table 12), SNX-111 (0.1 μg) enhanced the effects of sub-maximal doses of morphine, whereas SNX-124 (0.1 μg) had no effect. SNX-

25   183 (1 μg) showed a small but measurable effect, but this effect was not significant when compared to control. SNX-236, a TVIA-derivative shown in Figure 2 was also found to be analgesic in the Rat tail-flick assay.

30

39

## Table 11

### Effect of SNX-185 ± Morphine on Latency Percent Effect in Rat Tail-Flick Assay

| SNX-185 Dose (µg) | n | Time After Injection (min) % Effect (± SE) | | Morphine Dose (µg) | Time After Injection (min) % Effect (± SE) | |
|---|---|---|---|---|---|---|
| | | 25 | 45 | | 25 | 45 |
| 0 | 6 | 20 ± 16 | 18 ± 12 | 0.2 | 58 ± 22 | 66 ± 17 |
| 0.5 | 7 | 29 ± 13 | *57 ± 13 | 0.2 | 98 ± 2 | 92 ± 5 |
| 0 | 8 | 16 ± 15 | 12 ± 14 | 0.1 | 61 ± 15 | 75 ± 11 |
| 0.5 | 9 | 0 ± 4 | 13 ± 11 | 0.1 | 60 ± 17 | 51 ± 17 |
| 0 | 5 | 9 ± 5 | 8 ± 3 | 0.1 | 45 ± 20 | 49 ± 16 |
| 0.5 | 5 | 12 ± 10 | 8 ± 3 | 0.1 | 79 ± 21 | 77 ± 21 |
| 1.0 | 4 | 33 ± 22 | 36 ± 20 | 0.1 | − | − |
| 0 | 8 | 1 ± 3 | 5 ± 2 | 0.05 | 7 ± 6 | 16 ± 11 |
| 0.5 | 8 | *27 ± 11 | 14 ± 7 | 0.05 | *44 ± 16 | 30 ± 18 |

*$p < .05$ vs. Control.

## Table 12

### Effect of Coadministration of Conopeptides and Morphine on Analgesia (% Effect) in Rat Tail-Flick Assay

| | Time After Injection | | | |
|---|---|---|---|---|
| | 15 | 30 | 45 | MPE |
| Morphine, 0.05 µg (n=14) | | | | |
| Mean SE | 15 7 | 13 6 | 7 5 | 23 8 |
| SNX-111, 0.1 µg + morphine (n=13) | | | | |
| Mean SE | 12 9 | 33 10 | 38 12 | 42 11 |
| SNX-124, 0.1 µg + morphine (n=14) | | | | |
| Mean SE | -7 5 | 5 6 | 11 11 | 14 8 |
| SNX-183, 1.0 µg + morphine (n=14) | | | | |
| Mean SE | 18 6 | 31 8 | 25 6 | 39 7 |

$p < .05$ vs. Morphine

40

An _in vivo_ test of analgesic potency is the rat
formalin test.  Briefly, in this test, a standard dose
of formalin is injected into the rat paw, and flexions
of the paw are quantitated.  Typically in this assay a
5    biphasic response pattern is observed, with numerous
responses observed during the period 5 min. after
injection (Phase 1) and a second phase (Phase 2) which
occurs during the period about 10-60 minutes following
injection (Fig. 16).  Quantitation of responses during
10   each phase is made by calculation of area under the
curve of flinches/min. as described in Example 8.

Figure 17 shows results of experiments in which
SNX-111, SNX-185 and SNX-231 were tested for effects on
the formalin response in rats.  From the curves shown,
15   doses which resulted in approximately 50% inhibition in
Phase 1 and Phase 2 responses were determined (Table
13).  As shown in Figure 16, administration of SNX-111
and SNX-185 each resulted in dose dependent inhibition
of both Phase 1 and Phase 2 response evoked by
20   formalin.  SNX-231 was without effect at the doses
employed in the assay.

### Table 13

25   $ED_{50}$ Values for Intrathecal Administration of
Conopeptides on Phase 1 and Phase 2
of the Formalin Test

| Drug | N | Phase 1 | Phase 2 |
|------|---|---------|---------|
| SNX-111 | 21 | 0.009 $\mu$g | 0.013 $\mu$g |
| SNX-185 | 20 | 0.02 $\mu$g | 0.05 $\mu$g |
| SNX-231 | 12 | >1.0 $\mu$g | >1.0 $\mu$g |

Analgesic potency of conopeptides can also be
tested in animal models of neuropathic or neurogenic

41

pain. One such model resembles the human condition termed causalgia or reflex sympathetic dystrophy (RSD) secondary to injury of a peripheral nerve. This condition is characterized by hyperesthesia (enhanced

5    sensitivity to a natural stimulus), hyperalgesia (abnormal sensitivity to pain), allodynia (widespread tenderness, characterized by hypersensitivity to tactile stimuli), and spontaneous burning pain. In humans, the pain induced by neuropathy tends to be

10   chronic and may be debilitating. Treatment may involve repeated sympathetic ganglionic blockade. Such blockade can be carried out surgically or using direct application of drugs, such as anesthetics or morphine, even when such drugs are ineffective systemically. In

15   accordance with the invention, analgesic omega conotoxin peptides are effective in providing relief of neuropathic pain, as described below.

Experiments carried out in support of the present invention were performed in a rat model of peripheral

20   neuropathy detailed in Example 9. Briefly, in the model used, rats are subjected to a surgical procedure, described by Kim et al. and Bennett et al., designed to reproducibly injure peripheral nerves (spinal nerves L5 and L6). These rats develop a hyperesthetic state,

25   which can be measured, using one or more paradigms known in the art. Here, allodynia was measured by stimulation of neuropathic rat hindlimb using wire hairs having graded degrees of stiffness. Analgesic compounds reverse the heightened sensitivity such

30   animals exhibit to the stimulus.

Results of animals treated with saline, 0.3, 1, or 3 $\mu$g of SNX-111 are shown in Figures 18 and 19. Data are expressed as percent maximum effect, where the maximum effect indicates a complete reversal of

35   surgically induced allodynia, or relative insensitivity

42

to stimulus (maximum equals 15 gram hair stimulus). A baseline of zero indicates a mean sensitivity to a wire hair graded at less than 3 grams. As shown in Figure 18, treatment of rats (n=6/treatment) with 1 or 3 μg
5    SNX-111 resulted in elevation of threshold response. Peak elevation of response due to drug treatment (reversal of allodynia) was observed by 30-60 minutes, and effects lasted in excess of 60 minutes.

Figure 19 summarizes and shows statistical
10   analyses, as detailed in Example 9, of the data shown in Figure 18. Also shown in Figure 18 is the response of animals treated with 10 μg morphine sulfate. In the study shown, treatment of rats with 3 μg SNX-111 or 10 μg morphine resulted in significant reversal of
15   allodynia in comparison to saline treated animals.

These results indicate that analgesic omega conotoxin peptides, exemplified by SNX-111, are capable of reversing the hyperesthetic effects induced by nerve damage.

20

III.  Summary
As indicated above, conopeptides such as MVIIA and TVIA, and their derivatives, have a number of peptide-specific binding/inhibitory activities, which include:
25        (1)  high-affinity binding to the MVIIA binding site of neuronal cells;
          (2)  inhibition of norepinephrine release selectively in central nervous system neuronal cells;
          (3)  inhibition of voltage-gated calcium channels
30   selectively in neuronal tissue, as evidenced by the inhibition of electrically stimulated contraction of the guinea pig ileum; and
          (4)  Inhibition (blockage) of membrane currents associated with N-type or omega HVA neuronal calcium
35   channels in an isolated cell system, such as the mouse

43

neuroblastoma cell line;

Previously it has been shown (co-owned U.S. Patent No. 5,051,403) that conopeptides having defined binding/ inhibitory activities are effective in reducing neuronal damage related to an ischemic condition in mammals. The binding/inhibitory activities of conopeptides effective in such treatment include:

(a) high-affinity binding to the MVIIA binding site; and

(b) selective inhibition of norepinephrine release in central nervous system neuronal cells.

Two conopeptides which have these characteristic activities, and which have been shown effective in reducing post-ischemia neuronal damage, are conopeptides MVIIA and TVIA.

In the Section above, it was shown that conopeptides, such as MVIIA and TVIA, which have defined binding/inhibitory activities, are effective in producing analgesia and in enhancing analgesia by opiates. The important binding/inhibitory activities are:

(a) high-affinity binding to the MVIIA binding site; and

(b) inhibition of voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the inhibition of electrically stimulated contraction of the guinea pig ileum.

The following examples are intended to illustrate various characteristics of the method of the invention, but are in no way intended to limit the scope of the invention.

44

## Example 1
### Calcium-Channel Antagonist Activity:
### Inhibition of Ionic Currents

Ionic currents through calcium channels were
examined in cells that were voltage-clamped by a single
patch-clamp electrode.   These whole-cell patch-clamp
studies were performed mainly on N1E115 mouse
neuroblastoma cells, although a variety of cell types,
including human neuroblastoma cell line IMR-32, have
been examined.

A.  Current Measurement Methods

Most measurements were obtained using a bath
saline that allowed examination of the calcium currents
in the absence of other ionic currents.   These
solutions contained 80 mM NMDG (as a sodium
replacement), 30 mM TEACl (to block potassium
currents), 10 mM $BaCl_2$ (as a charge-carrier through the
calcium channels), and 10 mM HEPES at pH 7.3.  Some
solutions also contained 2 mM quinidine (to block
potassium currents) and 3 $\mu$M tetrodotoxin (to block
sodium currents).  Normal bath saline was (mM): 140
NaCl, 10 glucose, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10mM HEPES
pH 7.3.  Intracellular solutions contained 150 mM CsCl,
0.5 mM $CaCl_2$, 5 mM EGTA, 5 mM $MgCl_2$, 2 mM $K_2ATP$ at pH
7.3-7.4.  Bath saline and all internal solutions were
filtered before use.

Pipets were made from Corning 7052 glass (Garner
Glass Company, Claremont, CA 91711), coated with
Sylgard (Dow Corning, Midland, MI 48640) and
fire-polished before use.  Bubble numbers were
typically 5 to 6, with pipet resistances typically 2-5
MOhms.  Corning 8161, Kimble, and other glasses were
also used without noticeable effect on the calcium cur-
rents observed.

45

Recordings were carried out at room temperature
with an Axopatch 1-C amplifier (Axon Instruments,
Foster City, CA 94404) and analyzed with pCLAMP
software (Axon Instruments). Data were filtered at
5    1000 Hz for a typical sampling rate of .1 kHz; in all
cases data were filtered at a frequency at most 1/5 of
the sampling rate to avoid biasing. Data were col-
lected on-line by the software. Analysis was performed
on-screen with print-out via a Hewlett-Packard LaserJet
10   Printer (Hewlett-Packard, Palo Alto, CA 94306).
The typical experiment was conducted as follows:
after seal formation followed by series resistance
compensation and capacitative transient cancellation,
a voltage clamp protocol was performed wherein the cell
15   potential was stepped from the holding potential
(typically -100 mV) to test potentials that ranged from
-60 mV to +20 mV in 10 mV increments. The cell was
held at the holding potential for 5 seconds between
pulses.    Protocols starting from other holding
20   potentials usually covered the same range of test
potentials.

B.    Current Inhibition Measurement
Figure 3 shows calcium current traces from an N1E-
25   115 mouse neuroblastoma cell. The figure is read from
left to right in time, with downward deflections of the
trace indicating positive current flowing into the
cell. Currents were elicited by a voltage step from
100 mV to -10 mV. The cell was bathed in saline with
30   sodium replaced by NMDG and 10 mM $Ba^{++}$ instead of 2 mM
$Ca^{++}$. Potassium currents were blocked by TEA in the
bath and $Cs^{+}$ in the pipet solution.
The three traces in Figure 3, labeled B-D, show
decreasing calcium currents, with increasing MVIIA
35   omega-conopeptide concentrations of 10 nM (3B), 50 nM

46

(3C), and 200 nM (3D).

The response of voltage-gated calcium current to increasing dosages of OCTs MVIIA and GVIA are shown in Figure 4.  The calculated $IC_{50}$ is approximately 10 nM

5    for GVIA and 100 nM for MVIIA.  These values indicate extremely high specificity of the peptides for their site of action.

Table 1 compares $IC_{50}$ values for GVIA, MVIIA, SVIB and SVIA OCTs.  Whereas OCT GVIA and OCT MVIIA show 50%

10   inhibition of the measured calcium current at nanomolar concentration range, $IC_{50}$ values for OCT SVIB and OCT SVIA were not measurable within the range of concentrations tested, and are therefore listed as having $IC_{50}$ values above the micromolar concentrations

15   indicated.


## Example 2
### Synaptosomal Membrane Preparations

A.   Mammalian-Brain Synaptosomes and Synaptosomal

20   Membranes.

Synaptosomes were prepared from rat whole brain or hippocampal region of brain.  Rats were sacrificed, and forebrains were removed and transferred to 10 ml ice-cold 0.32 M sucrose containing the following

25   protease inhibitors (PI): 1 mM EGTA; 1 mM EDTA; 1 uM pepstatin; 2 uM leupeptin.  Brains were homogenized using a motor-driven Teflon-glass homogenizer (approx. 8 passes at 400 rpm). Homogenates from 4 brains were pooled and centrifuged at 900 xg for 10 minutes at 4

30   degrees. Supernatants were then centrifuged at 8,500 xg for 15 minutes.  Resulting pellets were resuspended in 10 ml each ice-cold 0.32 M sucrose plus PI with vortex mixing.  The suspension was then centrifuged at 8,500 xg for 15 minutes. Pellets were resuspended in 20 ml

35   ice-cold 0.32 M sucrose plus PI.  The suspension (5

47

ml/tube) was layered over a 4-step sucrose density gradient (7ml each: 1.2 M sucrose, 1.0 M sucrose, 0.8 M sucrose, 0.6 M sucrose; all sucrose solutions containing PI). Gradient tubes were centrifuged in a swinging bucket rotor at 160,000 xg for 60 minutes at 4 degrees. The 1.0 M sucrose layer plus the interface between the 1.0 and 1.2 M sucrose layers were collected and diluted with ice cold deionized water plus PI to yield a final sucrose concentration of 0.32 M. The resulting suspension was centrifuged at 20,000 xg for 15 minutes. Pellets were then resuspended in 5 ml ice-cold phosphate buffered saline plus PI. The resulting rat brain synaptosomes were then aliquoted and stored in a liquid nitrogen containment system.

Prior to use in binding assays, synaptosomes were thawed and diluted with 3 volumes of ice cold deionized water plus PI. This suspension was homogenized using a PT 10-35 Polytron (setting 6) for two 10-second bursts. The homogenate was centrifuged at 40,000 xg for 20 minutes at 4 degrees. The resulting pellets were resuspended in about 5 ml of ice cold phosphate buffered saline plus PI. The resulting brain synapto-somal membrane preparation was aliquoted and stored at -80°C until use. Protein concentration of the membrane preparation was determined using Bradford reagent (BioRad), with bovine serum albumin as standard.

B. Electric organ synaptosomes

Electric organ synaptosomes were prepared by dissection from marine electric rays (Ommata dyscopyge or Narcine brasiliensis) that had been stunned with 0.25 g/liter tricaine HCl and cooled to 4°C immediately prior to dissection. All subsequent manipulations were carried out at 0-4°C whenever possible. Organs were diced and homogenized for 4 15-second periods in a

48

Waring blender with an equal weight of synaptosome buffer (SB) (20 mM HEPES, Ph 7.2, 280 mM NaCl, 3mM KCl, 1.8 mM $MgCl_2$, 300 mM urea, 100 mM sucrose, 5.5 mM glucose plus protease inhibitors), (1 mM EGTA, 1 $\mu M$ pepstatin, 2 $\mu M$ leupeptin, 1 $\mu g/ml$ aprotinin and 0.1 mg/ml bacitracin).

The homogenate was filtered through cheesecloth and centrifuged at 30,000 x g for 15 min. The supernatant was discarded and each pellet was taken up in 10 ml synaptosome buffer plus protease inhibitors. The resuspended pellets were combined and further disrupted with 5 strokes of a Teflon pestle in a glass homogenizer set at 400 rpm. The resulting suspension was centrifuged at 30,000 x g for 15 min. The supernatant was discarded and the pellet resuspended in approximately 5 ml of SB with protease inhibitors using a Teflon-glass homogenizer. This homogenate was layered onto six 32 ml 3-20% Ficoll gradients in SB (no protease inhibitors) and centrifuged at 100,000 x g for 1 hour in a swinging bucket rotor. The synaptosome band (the first band below the buffer-gradient interface) of each gradient was aspirated off and diluted 2:1 with synaptosome buffer with protease inhibitors. The diluted synaptosome suspension was pelleted at 30,000 x g for 15 min and resuspended in synaptosome buffer and refrigerated, for use in ATP release assays within 2-3 days of preparation. For binding experiments, aliquots were frozen at -160°.

Example 3

Omega-conopeptide Binding to Omega-conopeptide Binding Sites in Synaptosomal Membranes

A.    Saturation Binding Assay

MVIIA OCT was radiolabeled with ^{125}I-iodine by

reaction with Iodogen™, essentially according to the method of Ahmad and Miljanich. Following the Iodogen reaction, the peptide solution was chromatographed by HPLC through a C-8 reversed phase column and eluted
5   with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in water/acetonitrile (40:60 vol/vol). The major peak of radioactivity following the underivatized MVIIA OCT was collected.

The binding constant ($K_d$) for $[^{125}I]$-MVIIA OCT to
10  rat brain synaptosomal membranes was determined by a saturation binding method in which increasing quantities of $[^{125}I]$ MVIIA OCT were added to aliquots of a synaptosomal membrane preparation (10 ug membrane protein, suspended in binding buffer consisting of 20
15  mM HEPES, pH 7.0, 75 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, $2 \mu M$ leupeptin, .035 $\mu g/ml$ aprotinin, and 0.1% bovine serum albumin (BSA), in a total volume of 0.5 ml). Binding at each concentration of labeled compound was determined in the absence and presence of 1 nM
20  unlabeled MVIIA OCT to determine specific binding (as described in part B, below). The amount of labeled peptide specifically bound at each concentration was used to determine $B_{max}$, the concentration of specific binding sites on the synaptosomes, and $K_d$, following
25  standard binding analysis methods (Bennett). Figure 6A shows a saturation binding curve of $[^{125}I]$MVIIA to rat synaptosomal membranes. Figure 6B shows a Scatchard transformation of the data, from which a calculated $K_d$ of about 10 pM is determined.
30  B.    Reversibility of Binding

Rat brain synaptosomal membranes were incubated with a concentration of radiolabeled ligand approximating the $K_d$ of the ligand for its binding site, for a period of time sufficient to achieve
35  equilibrium binding. A high concentration of unlabeled

50

ligand was then added to the mixture, and the
incubation continued. At time intervals, samples of
the mixture were tested for binding of radiolabeled
compound. As shown in Figure 7, SNX-111 exhibited
5    reversible binding with a dissociation half-time of
about 2 min. Likewise, SNX-183 binding exhibited
reversible binding with a dissociation half-time of
about 5 min. In contrast, radiolabeled SNX-124 showed
no dissociation from its binding site over the time
10   period studied (60 min).


     C.   Competitive Displacement Binding Assay
          1. Competitive Displacement of OCT MVIIA
          Rat brain synaptosomal membranes prepared as
15   described in Example 2 were suspended in a binding
buffer consisting of 20 mM HEPES, pH 7.0, 75 mM NaCl,
0.1 mM EGTA, 0.1 mM EDTA, 2 $\mu$M leupeptin, .035 $\mu$g/ml
aprotinin, and 0.1% bovine serum albumin (BSA). [$^{125}$I]-
MVIIA (SNX-111) OCT (25-30,000 cpm, approximately 1500-
20   2000 Ci/mmol) and test compound were aliquoted into
polypropylene tubes, in the absence or presence of 1 nM
MVIIA (SNX-111) OCT to determine non-specific binding.
The membrane suspension was diluted and aliquoted last
into the test tubes, such that each assay tube
25   contained 10 $\mu$g membrane protein and the total volume
was 0.5 ml. After incubation for 1 hour at room
temperature, tubes were placed in an ice bath, then
filtered through GF/C filters (Whatman), which were
pre-soaked in 0.6% polyethyleneimine and prewashed with
30   wash buffer (20 mM HEPES, pH 7.0, 125 mM NaCl, 0.1%
BSA) using a Millipore filtration system. Just prior
to filtration, each assay tube received 3 ml ice-cold
wash buffer. The filtered membranes were washed with
two 3 ml volumes of ice-cold wash buffer, dried, and
35   filter-bound radioactivity was measured in a Beckman

51

gamma counter (75% counting efficiency).

Representative displacement binding curves for rat
brain synaptosomal membranes are illustrated in Figure
8.    IC$_{50}$ values were computed from line fit curves
5    generated by a 4-parameter logistic function.  These
values represent the concentration of test compound
required to inhibit by 50% the total specific binding
of [$^{125}$I]-MVIIA (SNX-111) OCT to rat brain synaptosomal
membranes, where specific binding is defined as the
10   difference between binding of [$^{125}$I]-MVIIA (SNX-111) OCT
in the absence and presence of excess (1 nM) unlabelled
MVIIA OCT.  Non-specific binding is that binding of
radiolabeled compound which is measured in the presence
of excess unlabeled MVIIA OCT.  Such values serve as
15   approximations of the relative affinities of a series
of compounds for a specific binding site.
2. Competitive Displacement of OCT SVIB

Rat brain synaptosomal membranes were prepared as
described in Example 3.  OCT SVIB was radiolabeled by
20   iodination with $^{125}$I-iodine by the Iodogen reaction,
described in Example 4.  Displacement binding of
radiolabeled SVIB on rat brain synaptosomal membranes
was carried out as in Example 4B.  SVIB displacement
curves for several of the omega-conopeptides assayed is
25   shown in Figure 9.  IC$_{50}$ values and relative potency
values were calculated as described below.  Table 4
shows   the   relative   potency   values   for   omega-
conopeptides  examined,  and  the  ratio  of  relative
potencies of the compounds for the OCT MVIIA site and
30   to the SVIB binding site.

The binding constant (K$_i$) for each test substance
was   calculated   using   non-linear,   least-squares
regression analysis (Bennett & Yamamura) of competitive
binding data from 2 assays performed in duplicate on
35   separate occasions.  The relationship between K$_i$ and

52

IC$_{50}$ (concentration at which 50% of labeled compound is displaced by test compound is expressed by the Cheng-Prusoff equation:

$$K_i = IC_{50}/(1 + [L]/K_d)$$

where IC$_{50}$ is the concentration of test substance required to reduce specific binding of labeled ligand by 50%; [L] is the concentration of [$^{125}$I]-MVIIA (SNX-111) OCT used in the experiment; and K$_d$ is the binding constant determined for binding of [$^{125}$I]-MVIIA (SNX-111) OCT to rat brain synaptosomal membranes in saturation binding experiments.  Table 3 summarizes computed IC$_{50}$ for various omega-conopeptides for the MVIIA binding site of rat brain synaptosomal membranes.

Relative potency for displacement of binding is calculated as a ratio of the IC$_{50}$ of the test compound and the IC$_{50}$ of the reference compound.  The reference compound is generally the unlabeled equivalent of the labeled ligand.  Calculation of relative potency is as follows:

[log (relative potency)]=log (IC$_{50(ref)}$) -log(IC$_{50(test)}$)

Relative potency values for binding at OCT MVIIA (SNX-111) and OCT SVIB (SNX-183) sites are listed in Table 3.

Example 4
Crosslinking of [$^{125}$I]-SNX-111 and [$^{125}$I]-SNX-183 to their polypeptide receptors.

A.  SDS-gel electrophoretic analysis of rat hippocampal synaptosomal membrane polypeptides chemically crosslinked with A. [$^{125}$I]-SNX-111 and B. [$^{125}$I]-SNX-183.  Both radioactive ligands (1nM) were incubated with rat hippocampal synaptosomal membranes in the absence (middle lanes) or presence (right

53

lanes) of excess non-radioactive peptide at 1000
times the IC50 for binding (i.e., 10 nM for SNX-111
and 1 mM for SNX-183) and crosslinking was achieved
by the addition of N-hydroxysuccinimide (NHS) and the
5    water-soluble carbodiimide, EDC (45).. The left lanes
are controls to which EDC and NHS were not added.
B.   Displacement of crosslinked $^{125}$I-SNX-183 by
SNX-111
     [$^{125}$I]-SNX-111 (A) or [$^{125}$I]-SNX-183 (B)
10   crosslinked to the 210kDa polypeptide(s) in rat
hippocampal synaptosomal membranes were displaced by
increasing concentrations of non-radioactive SNX-111
and SNX-183 (45). As expected, the site 1-specific
ligand [$^{125}$I]-SNX-111 was displaced monotonically by
15   both SNX-111 and SNX-183. In contrast, displacement
of [$^{125}$I]-SNX-183 by SNX-111 is biphasic with IC50's
similar to its IC50's for binding to site 1 and site
2. The amount of radioiodinated peptide incorporated
into the 210kDa band of crosslinked hippocampal
20   synaptosomal membranes exposed to varying
concentrations of competing peptides was estimated by
scanning densitometry. The optical density of the
210kDa band in the sample without added competing
peptide was taken as 100%. Curves were fit to the
25   data as described above.

## Example 5

### Localization of OCT binding sites in Neuronal Tissue
### by Receptor Autoradiography

     Adult male (Fischer or Sprague-Dawley, 250-300
30   g) were euthanized with carbon-dioxide, and whole
brains were dissected out of the skull and rapidly
frozen in iso-pentane pre-cooled on frozen carbon
dioxide. The frozen brains were stored at -80°C and
used within a week.
35       Coronal sections (20 $\mu$ thick) were obtained by

54

slicing (at -10°C-15°C) through the frozen brain
using a cryostat microtome.  The sections were thaw-
transferred onto glass slides precoated with gelatin.
Glass slides with the frozen sections were stored

5   at -80°C and used within a week.  Binding of [$^{125}$I]
MVIIA was performed at room temperature.  Each brain
section was incubated for 40 min. with 250 $\mu$l of
binding buffer: (HEPES/NaOH (20 mM, pH 7.5), EDTA
(0.1 mM), EDTA (0.1mm) leupeptin (2 $\mu$M), Aprotinin

10  (0.63 mg/ml), 1.5% BSA (RIA Grade), and [$^{125}$I] MVIIA
(100-150 pM).  To determine the proportion of non-
specific binding selected adjacent brain sections
were incubated with an excess of unlabelled peptide
(25 nm).

15          After the incubation, binding buffer was
carefully poured onto blotting paper and the slides
transferred to a glass slide holder.  Unbound [$^{125}$I]
MVIIA was washed away by serially passing the slides
through four dishes of washing buffer at room

20  temperature for a total washing time of 16 min.
Washing buffer contained HEPES/NaCH (50 ml9, pH 7.5),
NaCl (170 mM), BSA (RIA grade 1g/L) and Triton X-100
(0.05%).  After the final wash, the slides were
dipped quickly five times in water and dried with a

25  blow-dryer.
            Dried slides were exposed to XAR-2 film,
overnight at room temperature and developed.  The
developed images were examined wither directly or by
computer assisted image analyzer.  The assignment of

30  binding to specific neuroanatomical sites was made
using an anatomical atlas_ of rat brain (Paxinos).
            Autoradiograms show_the distributions of
[$^{125}$I]-SNX-111 (A,B,C,D) and [$^{125}$I]-SNX-183 (E,F,G,H)
binding to coronal rat brain sections.  Labeling in

35  the presence of excess non-radioactive SNX-111 (C,D)

55

or SNX-183 (G,H) shows that non-specific labeling is
negligible. Rostral sections (A,C,E,G) and caudal
sections (B,D,F,H) are each adjacent or
near-adjacent. "CA" indicates the $CA_3$ region of the
5    hippocampus and "SN" indicates the substantia nigra.

## Example 6
### Inhibition of Neurotransmitter Release
A. Inhibition of Norepinephrine release
10        Inhibitory constants (IC50's) reflecting the
potency of SNX-111 and SNX-183, for blocking the
$K^+$-evoked release of exogenous, loaded
$[^3H]$-norepinephrine from rat hippocampal slices were
determined. Freshly dissected hippocampal slices in
15   oxygenated buffered saline were loaded with
[3H]-norepinephrine and washed three times. Slices
were then exposed to buffered saline (containing 3.3
mM $K^+$) for 1.5 minutes and the supernatants
containing released basal norepinephrine were
20   collected for scintillation counting. The slices
were then depolarized by exposure to buffered saline
containing 30mM $K^+$ for 1.5 minutes and the
supernatants, containing evoked norepinephrine, were
also collected for scintillation counting. Slices
25   were exposed to the desired concentration of peptide
in all solutions from the time of loading with
norepinephrine to the end of the experiment (about 2
hours). The data points are the differences of the
means of 7 basal determinations and 7 evoked
30   determinations at each drug concentration. Release
in the absence of drug is taken as 100 per cent and
the remaining points are scaled accordingly. The
error bars are the standard errors of the means of
the differences. Curves of best fit and the
35   corresponding IC50's were derived. The single IC50

56

for SNX-111 is correlated with binding to site 1
calcium channels; the two IC50s for SNX-230 are for
inhibition associated with binding to site 1 calcium
channels (65nM) and to site 2 calcium channels (0.02

5   nM); the apparent single IC50 for SNX-183 is presumed
to reflect binding to both site 1 and site 2 calcium
channels with about equal affinity (see text).
Evoked release in the absence of $Ca^{++}$ in the buffer
was equal to basal release (data not shown); thus all

10  release shown is calcium-dependent release.
B.   Inhibition of ATP release from Electric Organ Sy-
naptosomes
    Synaptosomes were prepared substantially as
described in Example 3A.  The diluted synaptosome

15  suspension from the final centrifugation step was
pelleted at 30,400 x g for 15 min and resuspended in
1 ml of synaptosome buffer (with the inclusion, for
some experiments, of 1% BSA to enhance stability of
the synaptosomes).  This final synaptosome prepara-

20  tion was stored at 0°C and used for ATP release
experiments within 30 hours.  Storage for longer
periods resulted in the almost complete loss of
depolarization-dependent ATP release activity.
    Luminometry was performed according to published

25  method (Morel, Schweitzer).  Into a 5 ml polypropy-
lene test tube were mixed 465 $\mu$l synaptosome buffer,
5 $\mu$l of 5 $\mu$g/ml luciferin in PSB, 20 $\mu$l firefly
lantern extract (1 Sigma FLE-50 bottle reconstituted
in 1 ml PSB and spin-dialyzed through 3 ml of

30  Sephadex G-25 pre-equilibrated in PSB), 5 $\mu$l 100 mM
$CaCl_2$, and 5 $\mu$l synaptosome suspension (5-7 mg/ml
protein, excluding BSA).  The tube was placed in the
chamber of a custom-built luminometer and the light
output produced by extracellular ATP was continuously

35  monitored by a chart recording of the voltage

57

generated by the photomultiplier tube.  Exocytotic
release of ATP was evoked by injecting 0.5 ml of high
K+ buffer (synaptosome buffer with equimolar replace-
ment of Na+ by K+) into the reaction mixture in the
5     luminometer.

ATP release was quantitated by comparing the
peak heights of unknowns with the heights of peaks
generated by ATP standards that were injected into
each reaction mixture at the end of each trial.  Over
10    the range investigated, light output was linear with
respect to the amount of ATP injected.  $IC_{50}$ values
were calculated from the dose-dependent ATP
inhibition curves, and are reported in Table 6.

15    C.    Inhibition of Dopamine release from rat striatal
slices

Slices (0.3 x 0.3 x 1.5 mm) were prepared from
rat striatum, and were pre-loaded with radiolabeled
(tritiated) dopamine.  Slices were perfused for 45
20    minutes in Krebs Ringer Bicarbonate buffer
(oxygenated) as bathing medium.  Release of
neurotransmitter was stimulated by adding to the
perfusion medium KCl at a concentration ranging
between 4.8 and 15 mM, for a period of one minute.
25    The first such exposure was termed S1.  Perfusion
with bathing medium was continued.  Test compound(s)
were introduced into the perfusion medium 20 minutes
before the second stimulation (S2), which was done
identically to S1.  The ratio of S2/S1 was calculated
30    to determine drug effects.  A drug was considered to
block release if S2/S1 was significantly less than
unity.

D.    Inhibition of Acetylcholine Release from
35    Striatal Slices.

58

Release of acetylcholine was measured as
described above in part C for dopamine release,
except that slices were pre-loaded with radiolabelled
choline instead of dopamine.

5   E.   Inhibition of Electrically Stimulated
Contractions of Guinea Pig Ileum

Guinea pigs (300-400 gms) were decapitated and
the ileum removed.  A section of ileum about 6 cm
from the caecum was placed immediately into Krebb's
10  modified buffer maintained at 37°C in a water bath,
and aerated with a mixture of 95% $O_2$ and 5% $CO_2$.  The
buffer contains: KCl, 4.6 mM; $KH_2PO_4$, 1.2 mM; $MgSO_4$,
1.2 mM; Glucose, 10.0 mM; NaCl 118.2 mM; $NaHCO_3$, 24.8
mM; $CaCl_2$, 2.5 mM.

15  Small pieces of ileum were cut and pulled over a
glass pipette, scored and the logtudinal muscle
removed.  Each piece was attached to an electrode at
one end and to a force transducer at the other end.
The preparation was lowered into an organ bath
20  maintained at 37°C and aerated with $O_2$:$CO_2$.  The
resting tension was set at 1 gm, and the tissue was
stimulated at 30-50V with a duration of 4.5 msec per
stimulation.

Baseline responses (contractions) were recorded.
25  for 10-15 min. and aliquots (100 ml) of drug were
added to the bath until inhibition occurred.
Following testing, tissues were washed until original
response magnitude was achieved.


30  F.   Inhibition of amino acid neurotransmitter release
from rat brain slices

Male Sprague-Dawley rats were lightly
anesthetized with ether, decapitated, and the brains
removed to ice cold oxygenated basal medium (in mM:
35  NaCl; 118, KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; $KH_2PO_4$,

59

1.2; glucose, 11). Hippocampus and cerebral cortex
were further dissected from the brain and slices
(300-400 μm thick) were prepared using a γ McIlwain
Tissue Chopper at 4 degrees. Each slice was
5    preincubated at 37 degrees for 15 minutes. Buffer
was then replaced with an equal volume of either
basal medium or stimulation medium (in mM: NaCl; 88,
KCl, 30; CaCl$_2$, 1.3; MgSO$_4$, 1.2; KH$_2$PO$_4$, 1.2; glucose,
11). Incubation was then continued for 15 minutes.
10   Tubes containing slices were then centrifuged for 1
minute in a Beckman Microfuge. The supernatants were
collected and heated for 10 minutes at 100 degrees.
Aliquots (20ul) were used for analysis of amino acid
content using pre-column derivatization with o-
15   phthalaldehyde followed by HPLC as described by
Newcomb.

Figure 13B shows the effect of MVIIA OCT on K-
stimulated release of amino acid neurotransmitters
(Aspartate, GABA, glutamate) contrasted to effects on
20   serine, which is not a neurotransmitter. Significant
reductions in the amount of GABA and glutamate were
observed, as reported in Table 7.


Example 7
25   Rat Tail-Flick Assay for Analgesia
Male Sprague-Dawley rats (250-300g; Simonsen)
were implanted with intrathecal (i.t.) catheters,
which were inserted through the atlanto-occipital
membrane and threaded subdurally about 8 cm
30   therefrom. Animals were not used in experiments
until at least 2 days following implantation.
To perform the Tail-FLick test, a rat was
restrained in a plastic cone having openings at each
end, and was placed on a platform, positioned such

60

that its tail hung down from the platform in close
proximity to a heating bulb. Latency to flick the
tail away from the bulb was recorded. A trial
consisted of four such flicks at 1-2 min. intervals,
5   where the first latency time was generally not used,
and the three subsequent tests were averaged.
Latencies measured in the absence of analgesic
agent(s) were recorded for each rat as "Baseline
latency."
10      Rats were then removed from the restraining
cones, and injected (i.t.) with test compound in a
volume of 5 $\mu$l, followed by 10 $\mu$l saline. Animals
were subjected to post-drug trials at one or more
time intervals thereafter (usually 25 min and 45
15  min.), as described above.  In the cases where drug
enhancement was tested, test compound was first
injected, followed by tail-flick trials, to assess
the potency of the drug alone. Approximately 1 hour
later, a known analgesic, such as morphine, was
20  injected, and trials repeated.
        Drug effects were calculated as follows:

$$\% \text{ Effect} = 100 \times \frac{(\text{post-drug latency}) - (\text{baseline latency})}{(\text{maximum latency}) - (\text{baseline latency})},$$

where maximum latency was measured as experimental
25  cut-off time, the time beyond which the tail was not
allowed by the experimenter to be exposed to heat,
due to risk of burn to the animal.


                    Example 8
30            Rat Formalin Test for Analgesia
        Rats (male Sprague-Dawley, 275-300 g) were
implanted with lumbar intrathecal catheters under
halothane anesthesia. Catheters extended from the
cisterna to the rostral edge of the lumbar
35  enlargement. 3-5 days after implant, animals without

61

motor dysfunction were used.

Animals were examined for the effects of drugs
given in the formalin test, in which 50 ul of 5%
formalin was injected on the plantar surface of the
5      paw. The number of flexions of the paw were counted
at intervals after the injection of the formalin.
Drugs tested in this assay were dissolved in sterile
saline (0.9% NaCl) and injected in a volume of 10 ul
followed by 10 ul to clear the catheter.
10     Injection of formalin alone or with vehicle
(saline) resulted in a biphasic response pattern of
hind paw withdrawals (see Figure 16). The area under
the curve of the flinches/min was calculated for
phase 1 (time = 0-10 min) and phase 2 (10-60 min).
15     These values were plotted versus the intrathecal log
dose (ug) and the results are shown in Figure 17.


## Example 9
### Rat model of peripheral neuropathy
20     Male Sprague-Dawley rats (200-350 gm) were
prepared with chronic lumbar intrathecal catheters
inserted under halothane anesthesia (Yaksh and Rudy).
Animals were placed in a prone position and the left
paraspinal muscles were separated from the spinous
25     processes at the $L_4$-$S_2$ levels, as described by Kim et
al. The left L5 and L6 nerve roots were exposed and
tightly ligated with 6-0 surgical silk suture. The
rats were allowed to recover from anesthesia.
Allodynia was typically observed to occur beginning
30     1-2 days post-surgery and continuing for as long as
45 days.

For testing, animals were placed in plastic
cubicles with open wire mesh bottoms. Compound
dissolved in preservative-free saline solution was
35     administered in a volume of 10 $\mu$l through the

62

intrathecal catheter, followed by 10 μl saline to
flush the catheter line.  Animals were tested for
allodynia at various time points after drug
treatment, as described below.

5          To assess the threshold of a non-noxious
stimulus required to produce a left hind paw
withdrawal (allodynia), Von Frey hairs (ranging from
0.4-15 grams), were systematically applied to the
surgically treated hind paw.  Failure to evoke a
10   response was cause to test the next stiffer hair.
Evocation of a brisk withdrawal response was cause to
test the next lower stimulus intensity.  This
paradigm was repeated according to a statistical
method (Dixon) to define the 50% response threshold.
15   Allodynia was evidenced by a threshold less than 3
grams (referring to the hair stimulus intensity)
exhibited by all surgically treated animals.

          Results of animals treated with saline, 0.3, 1,
or 3 μg of SNX-111 are shown in Figures 18 and 19.
20   Data in Figure 17 are expressed as percent maximum
effect, where the maximum effect indicates a complete
reversal of allodynia, or insensitivity to stimulus
(maximum equals 15 gram hair cutoff).  A baseline of
zero indicates a mean sensitivity less than 3 grams.
25   As shown in Figure 18, treatment of rats
(n=6/treatment) with 1 or 3 μg SNX-111 resulted in
elevation of threshold response.  Peak effects were
observed by 30-60 minutes, and effects lasted in
excess of 60 minutes.

30          The results shown in Figure 18 were summarized
and subjected to statistical analyses (1-way ANOVA;
Games-Howell test) in Figure 19.  Also shown in
Figure 18 is the response of animals treated with 10
μg morphine sulfate.  In the study shown, treatment
35   of rats with 3 μg SNX-111 or 10 μg morphine resulted

63

in significant reversal of allodynia in comparison to
saline treated animals.

Animals were also observed for the appearance of
general motor dysfunction, as evidenced by inability
5    to ambulate symmetrically and for any other overt
signs of unusual activity, such as tremor.  No
effects on motor activity were observed in saline or
morphine-treated animals; a dose-dependent tremor
characteristic of SNX-111 administration was observed
10   in animals given SNX-111.

Although the invention has been described with
respect to particular embodiments, it will be
apparent to those skilled that various changes and
modifications can be made without departing from the
15   invention.

64

IT IS CLAIMED:

1.  A method of producing analgesia in a mammalian subject, comprising

administering to the subject, an omega conopeptide selected from the group consisting of TVIA (SNX-185), MVIIA (SNX-111) and derivatives thereof which are effective (a) to inhibit voltage-gated calcium channels selectively in neuronal tissue, as evidenced by the peptide's ability to inhibit electrically stimulated contraction of the guinea pig ileum, and (b) to bind to omega conopeptide MVIIA binding sites present in neuronal tissue.

2.  The method of claim 1, wherein the activities of the omega conopeptide in calcium-channel inhibition and in binding to the MVIIA binding site are within the ranges of such activities of omega-conotoxins MVIIA and TVIA.

3.  The method of claim 1, wherein the omega conopeptide is MVIIA or TVIA.

4.  The method of claim 1, wherein the omega conopeptide is SEQ ID NO: 30 (SNX-236).

5.  The method of claim 1, wherein the analgesia is produced in a subject exhibiting neuropathic pain.

6.  The method of claim 1, wherein the omega conopeptide is administered intrathecally.

7.  A method of enhancing the analgesic effect produced by administration of opiates to a mammalian

65

subject, comprising

co-administering to the subject, an omega
conopeptide selected from the group consisting of
TVIA (SNX-185), MVIIA (SNX-111) and derivatives
5    thereof which are effective (a) to inhibit voltage-
gated calcium channels selectively in neuronal
tissue, as evidenced by the peptide's ability to
inhibit electrically stimulated contraction of the
guinea pig ileum, and (b) to bind to omega
10   conopeptide MVIIA binding sites present in neuronal
tissue, at a dose effective to enhance the analgesic
effect of the opiate.

8.    The method of claim 5, wherein the
15   activities of the omega conopeptide in calcium-
channel inhibition and in binding to the MVIIA
binding site are within the ranges of such activities
of omega-conotoxins MVIIA and TVIA.

20        9.    The method of claim 5, wherein the omega
conopeptide is MVIIA or TVIA.

10.    The method of claim 5, wherein the omega
conopeptide is administered intrathecally.
25

11.    An omega conopeptide having the sequence:
SEQ ID NO: 30 (SNX-236).

```
                1      5       10      15      20      25      30

MVIIA/
SNX-     C K G K G A K C S R L M Y D C C T G S C - R - S G K - C
111

MVIIB/
SNX-     C K G K G A S C H R T S Y D C C T G S C N R - - G K - C
159

GVIA/
SNX-     C K S X G S S C S X T S Y N C C R - S C N X Y T - K R C - - - Y
124

GVIIB/
SNX-     C K S X G T X C S R G M R D C C T - S C L L Y S N K - C R R Y
178

RVIA/
SNX-     C K P X G S X C R V S Y N C C G - S C K S Y - N K K C G
182
```

Fig. 1

```
                    1      5         10        15        20        25      30

SVIA/
SNX-      C R S S S C G V T S I - C C - G R C - - Y R G K - C T
157

TVIA/
SNX-      C L S X G S S C S X T S Y N C C R - S C N X Y S - R K C R
185

SVIb/
SNX-      C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C
183

MVIIC/
SNX-      C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
230

SNX-      C K G K G A X C R K T M Y D C C S G S C G R - R G K - C
231
```

Fig. 1 (con't)

```
                 1        5         10        15        20        25
MVIIA
(SNX-111)        C K G K G A K C S R L M Y D C C T G S C R S G K C-NH2

SNX-190          - - - - A - - - - - - - - - - - - - - - - - - - NH2
SNX-191          - - A - - - - - - - - - - - - - - - - - - - - - NH2
SNX-193          - - - - - - - - - - - - - - - - - - - - - - - - G-OH
SNX-194          - - - - - - - - - - - -Nle- - - - - - - - - - - NH2
SNX-195          - - - - - - - - - - - - - - - - - - - - - - A - NH2
SNX-196   N-     - - - - - - - - - - - - - - - - - - - - - - - - G-OH
SNX-197   NG-    - - - - - - - - - - - - - - - - - - - - - - - - NH2
SNX-198          - - - - - - - - - - - - - - - - - - - - A - - - NH2
SNX-200          - - A - - - - - - - - - - - - - - - - - - - - - NH2
SNX-201          - - - - - - - - R K T G - - - - - - - - - - - - NH2
```

Fig. 2

SVIB
(SNX-183)    C K L K G Q S C R K T S Y D C C S G S C G R S G K C NH₂

SNX-202      - - - - - - - - - - - - S R L M - - - - - - - - NH₂

TVIA
(SNX-185)    C L S X G S S C S X T S Y N C C R S C N X Y S R K C R NH₂

SNX-207      - - - - - - - - - - R L M - - - - - - - - - - NH₂

SNX-236      - - - - - - - - - - R L M - - - - P - - - - - NH₂

Fig. 2 (con't)

# Fig. 3



200 pA

20 ms

# Fig. 4



CALCIUM CURRENT % CONTROL

CONCENTRATION, M

# Fig. 5A



4 pA

40 ms



# Fig. 5B

MINUTES + SNX-111

Fig. 5C

## Fig. 6A



SPECIFIC BINDING (pmoles)

MVIIA, pM



B / F

Bound (pmoles)

## Fig. 6B

Fig. 7

Fig. 10A

Fig. 10B

## Fig. 8A



## Fig. 8B

## Fig. 9A



## Fig. 9B

# Fig. 11A



# Fig. 11B

CAUDAL SECTIONS

SN

Fig. 12B

ROSTRAL SECTIONS

Fig. 12A

*111

Fig. 12D

Fig. 12C

*111
+
excess
111

CAUDAL SECTIONS

SN

Fig. 12F

ROSTRAL SECTIONS

Fig. 12E

*183

Fig. 12H

Fig. 12G

*183
+
excess
183
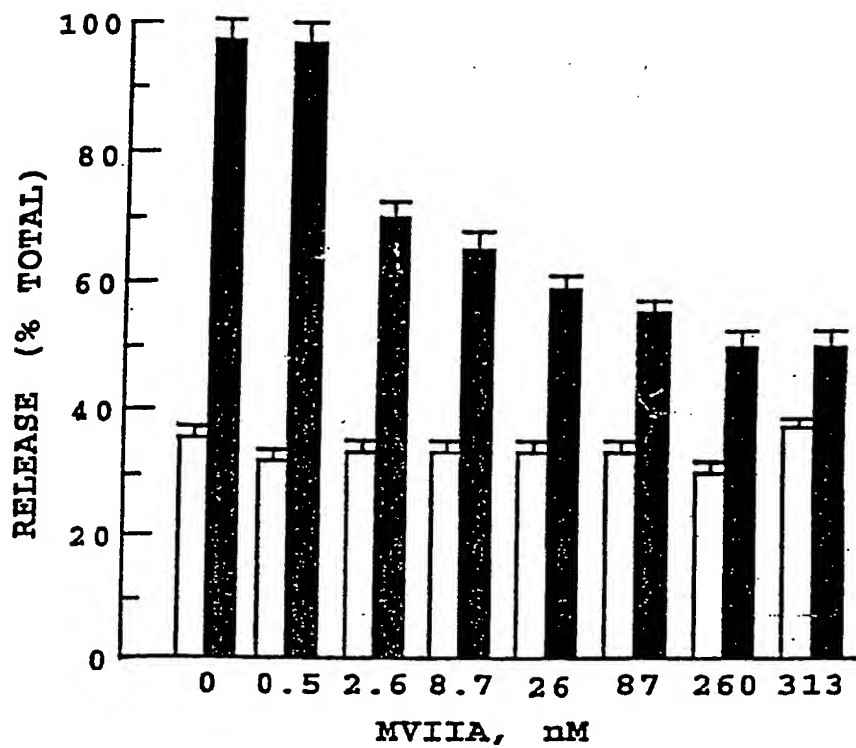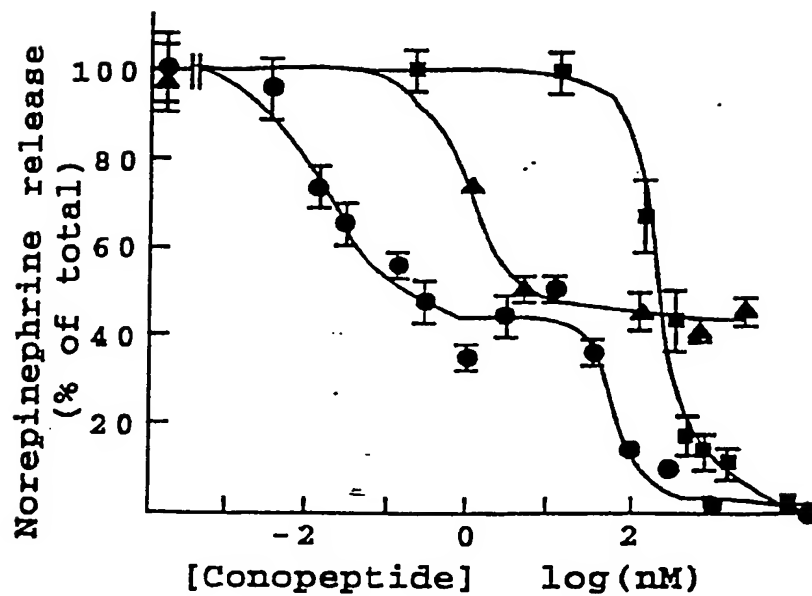
Fig. 13A



Fig. 13B

```
              1      5         10        15        20        25        30

I.
MVIIA         C K G K G A K C S R L M Y D C C T G S C - R - S G K - C
MVIIB         C K G K G A S C H R T S Y D C C T G S C N R - - G K - C

II.
TVIA          C L S X G S S C S X T S Y N C C R - S C N X Y S R K - C R
SNX-207       C L S X G S S C S R L M Y N C C R - S C N X Y S R K - C R
SNX-236       C L S X G S S C S R L M Y N C C R - S C N P Y S R K - C R

III.
RVIA          C K P X G S X C R V S S Y N C C S - S C K S Y - N K K C G
SVIA          C R S S G S X C G V T S I - C C - Y R G K - C T
GVIIA         C K S X G T X C S R G M R D C C T - S C L L Y S N K - C R R Y D
SVIB          C K L K Q S C R K T S Y D C C S G S C G R - S G K - C
MVIIC         C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
SNX-231       C K G K G A X C R K T M Y D C C S G S C G R - R G K - C
```

Fig. 14

Fig. 15



Fig. 16A

Fig. 16B



Fig. 16C

Fig. 17A



Fig. 17B

Fig. 18



Fig. 19

# INTERNATIONAL SEARCH REPORT

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/10;      A61K37/02

## II. FIELDS SEARCHED

### Minimum Documentation Searched⁷

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C07K ;    A61K |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

## III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

| Category° | Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹² | Relevant to Claim No.¹³ |
|---|---|---|
| P,X | EUROPEAN JOURNAL OF PHARMACOLOGY vol. 218, no. 1, 21 July 1992, pages 75 - 81 L. BASILICO ET AL. 'Influence of omega-conotoxin on morphine analgesia and withdrawal syndrome in rats' see results on pages 77-78 see discussion on pages 78-79 | 1,2,5-8 |
| P,X | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 89, no. 9, 1 May 1992, WASHINGTON US pages 3825 - 3829 K. MACKIE AND B. HILLE 'Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells' see page 3827, right column, paragraph 2 - page 3828, left column, paragraph 1; figure 4 | 7,8 |

-/--

° Special categories of cited documents :¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date.

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 14 APRIL 1993 | 27. 04. 93 |
| International Searching Authority | Signature of Authorized Officer |
| EUROPEAN PATENT OFFICE | FUHR C.K.B. |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT | (CONTINUED FROM THE SECOND SHEET) | |
|---|---|---|
| Category* | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| A | NEUROSCIENCE LETTERS<br>vol. 126, no. 1, 13 May 1991,<br>pages 67 - 70<br>M. OCANA AND J.M. BAEYENS 'Analgesic effects of centrally administered aminoglycoside antibiotics in mice'<br>see page 67, left column, paragraph 1 - right column, paragraph 1<br>see page 69, left column, paragraph 5 - right column, paragraph 2 | 1,5,6 |
| A | PAIN<br>vol. 41, no. 3, June 1990, AMSTERDAM, NETHERLANDS<br>pages 365 - 371<br>W.A. PRADO ET AL. 'Antinociception induced by intraperitoneal injection of gentamicin in rats and mice'<br>see page 369, right column, paragraph 3 | 1,5-7 |
| A | WO,A,9 107 980 (NEUREX CORPORATION)<br>13 June 1991<br>see page 11, line 5 - page 15, line 25;<br>claims 1-13; figures 1,2,14 | 1-3,11 |

**Box I    Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-10 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II    Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**        [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

## ANNEX TO THE INTERNATIONAL SEARCH REPORT
## ON INTERNATIONAL PATENT APPLICATION NO.

US 9211349
SA 68886

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

14/04/93

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9107980 | 13-06-91 | US-A- | 5051403 | 24-09-91 |
| | | AU-A- | 6964091 | 26-06-91 |
| | | US-A- | 5189020 | 23-02-93 |

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82